Q L

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 08 February 2000 (08.02.00)	
International application No. PCT/DK99/00382	**Applicant's or agent's file reference** 21134 PC 1
International filing date (day/month/year) 02 July 1999 (02.07.99)	**Priority date** (day/month/year) 03 July 1998 (03.07.98)
Applicant NILSSON, Dan et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

17 December 1999 (17.12.99)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Nestor Santesso
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* 21134 PC 1

Box No. I TITLE OF INVENTION

Method of preventing bacteriophage infection of bacterial cultures

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

Chr. Hansen A/S
Bøge Allé 10-12
2970 Hørsholm
Denmark

☐ This person is also inventor.

Telephone No.

Facsimile No.

Teleprinter No.

State *(that is, country)* of nationality: Denmark

State *(that is, country)* of residence: Denmark

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

NILSSON, Dan
Gefionsbakken 4A
3060 Espergærde
Denmark

This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: Denmark

State *(that is, country)* of residence: Denmark

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

Plougmann, Vingtoft & Partners
Sankt Annæ Plads 11
P.O. Box 3007
1021 Copenhagen K
Denmark

Telephone No.
+45 33 63 93 00

Facsimile No.
+45 33 63 96 00

Teleprinter No.

☐ **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Continuation of Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

If none of the following sub-boxes is used, this sheet should not be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

JANZEN, Thomas
Ingemannsvej 30, 3. tv.
1964 Frederiksberg
Denmark

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: Denmark

State *(that is, country)* of residence: Denmark

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

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Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

---

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

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Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

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☐ Further applicants and/or (further) inventors are indicated on another continuation sheet.

Box No.V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes; at least one must be marked)*:

Regional Patent

- ☒ AP ARIPO Patent: GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, SD Sudan, SZ Swaziland, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- ☒ EA Eurasian Patent: AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- ☒ EP European Patent: AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, CY Cyprus, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- ☒ OA OAPI Patent: BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, GW Guinea-Bissau, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

- ☒ AL Albania .....
- ☒ AM Armenia .....
- ☒ AT Austria ..and..utility..model...
- ☒ AU Australia .....
- ☒ AZ Azerbaijan
- ☒ BA Bosnia and Herzegovina .....
- ☒ BB Barbados
- ☒ BG Bulgaria .....
- ☒ BR Brazil .....
- ☒ BY Belarus .....
- ☒ CA Canada
- ☒ CH and LI Switzerland and Liechtenstein
- ☒ CN China .....
- ☒ CU Cuba .....
- ☒ CZ Czech Republic and.utility.model
- ☒ DE Germany..and.utility.model.
- ☒ DK Denmark..and.utility.model.
- ☒ EE Estonia...and.utility.model.
- ☒ ES Spain .....
- ☒ FI Finland...and.utility.model.
- ☒ GB United Kingdom
- ☒ GD Grenada
- ☒ GE Georgia .....
- ☒ GH Ghana .....
- ☒ GM Gambia
- ☒ HR Croatia .....
- ☒ HU Hungary .....
- ☒ ID Indonesia
- ☒ IL Israel .....
- ☒ IN India .....
- ☒ IS Iceland
- ☒ JP Japan .....
- ☒ KE Kenya .....
- ☒ KG Kyrgyzstan .....
- ☒ KP Democratic People's Republic of Korea .....
- ☒ KR Republic of Korea .....
- ☒ KZ Kazakhstan .....
- ☒ LC Saint Lucia
- ☒ LK Sri Lanka
- ☒ LR Liberia
- ☒ LS Lesotho .....
- ☒ LT Lithuania
- ☒ LU Luxembourg
- ☒ LV Latvia
- ☒ MD Republic of Moldova .....
- ☒ MG Madagascar .....
- ☒ MK The former Yugoslav Republic of Macedonia .....
- ☒ MN Mongolia
- ☒ MW Malawi .....
- ☒ MX Mexico .....
- ☒ NO Norway
- ☒ NZ New Zealand .....
- ☒ PL Poland .....
- ☒ PT Portugal .....
- ☒ RO Romania
- ☒ RU Russian Federation .....
- ☒ SD Sudan
- ☒ SE Sweden
- ☒ SG Singapore
- ☒ SI Slovenia .....
- ☒ SK Slovakia .and.utility.model.....
- ☒ SL Sierra Leone
- ☒ TJ Tajikistan .....
- ☒ TM Turkmenistan .....
- ☒ TR Turkey .....
- ☒ TT Trinidad and Tobago .....
- ☒ UA Ukraine .....
- ☒ UG Uganda .....
- ☒ US United States of America .....
- ☒ UZ Uzbekistan .....
- ☒ VN Viet Nam .....
- ☒ YU Yugoslavia .....
- ☒ ZW Zimbabwe .....

Check-boxes reserved for designating States (for the purposes of a national patent) which have become party to the PCT after issuance of this sheet:

- ☐ AE United Arab Emirates .....
- ☐ ZA.South.Africa .....
- ☐ .....

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)*

PLOUGMANN VINGTOFT & PARTNERS

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU 09 SEP. 1999

PCT

NOTIFICATION CONCERNING SUBMISSION OR TRANSMITTAL OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

To:

HEP/BH

PLOUGMANN, VINGTOFT & PARTNERS
Sankt Annæ Plads 11
P.O. Box 3007
DK-1021 Copenhagen K
DANEMARK

Date of mailing (day/month/year) 25 August 1999 (25.08.99)	
Applicant's or agent's file reference 21134 PC 1	**IMPORTANT NOTIFICATION**
International application No. PCT/DK99/00382	International filing date (day/month/year) 02 July 1999 (02.07.99)
International publication date (day/month/year) Not yet published	Priority date (day/month/year) 03 July 1998 (03.07.98)
Applicant CHR. HANSEN A/S et al	

1. The applicant is hereby notified of the date of receipt (except where the letters "NR" appear in the right-hand column) by the International Bureau of the priority document(s) relating to the earlier application(s) indicated below. Unless otherwise indicated by an asterisk appearing next to a date of receipt, or by the letters "NR", in the right-hand column, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).

2. This updates and replaces any previously issued notification concerning submission or transmittal of priority documents.

3. An asterisk(*) appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b). In such a case, the attention of the applicant is directed to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

4. The letters "NR" appearing in the right-hand column denote a priority document which was not received by the International Bureau or which the applicant did not request the receiving Office to prepare and transmit to the International Bureau, as provided by Rule 17.1(a) or (b), respectively. In such a case, the attention of the applicant is directed to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

Priority date	Priority application No.	Country or regional Office or PCT receiving Office	Date of receipt of priority document
03 July 1998 (03.07.98)	PA 1998 00878	DK	28 July 1999 (28.07.99)
06 July 1998 (06.07.98)	60/091,735	US	27 July 1999 (27.07.99)

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer

Carlos Naranjo

Telephone No. (41-22) 338.83.38

Box No. VI PRIORITY CLAIM ☐ Further priority claims are indicated in the Supplemental Box.

Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is: national application: country	regional application:* regional Office	international application: receiving Office
item (1) 3 July 1998	PA 1998 00878	Denmark		
item (2) 6 July 1998	US 60/091,735	USA		
item (3)				

☒ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office)* identified above as item(s): (1)

* *Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.*

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):*
ISA / EPO

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority):*
Date *(day/month/year)*: 13/11-98 Number: RS 101347 DK Country *(or regional Office)*: EPO

Box No. VIII CHECK LIST; LANGUAGE OF FILING

This international application contains the following number of sheets:

request	:	4
description (excluding sequence listing part)	:	25
claims	:	4
abstract	:	1
drawings	:	12
sequence listing part of description	:	2
Total number of sheets	:	48

This international application is accompanied by the item(s) marked below:

1. ☒ fee calculation sheet
2. ☒ separate signed power of attorney
3. ☐ copy of general power of attorney; reference number, if any:
4. ☐ statement explaining lack of signature
5. ☒ priority document(s) identified in Box No. VI as item(s): (2)
6. ☐ translation of international application into (language):
7. ☒ separate indications concerning deposited microorganism or other biological material
8. ☒ nucleotide and/or amino acid sequence listing in computer readable form
9. ☒ other *(specify)*: Copy of Standard search report

Figure of the drawings which should accompany the abstract:

Language of filing of the international application: English

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

Copenhagen, 2 July 1999
Plougmann, Vingtoft & Partners

Henry Søgaard

For receiving Office use only

1. Date of actual receipt of the purported international application:
2. Drawings: ☐ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:
4. Date of timely receipt of the required corrections under PCT Article 11(2):
5. International Searching Authority (if two or more are competent): ISA /
6. ☐ Transmittal of search copy delayed until search fee is paid.

For International Bureau use only

Date of receipt of the record copy by the International Bureau:

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

PLOUGHMANN VINGTOFT & PARTNERS

To:

PLOUGHMAN, VINGTOFT & PARTNERS A/S
Sankt Ann Plads 11
P.O. Box 3007
DK-1021 Copenhagen K
DANEMARK

21 SEP. 2000

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	19.09.00
Applicant's or agent's file reference 21134 PC 1	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/DK99/00382	02/07/1999	03/07/1998

Applicant
CHR. HANSEN A/S et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Vullo, C Tel.+49 89 2399-8061

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
21134 PC 1		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/DK99/00382	02/07/1999	03/07/1998
International Patent Classification (IPC) or national classification and IPC		
C12N1/20		
Applicant		
CHR. HANSEN A/S et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 5 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
17/12/1999	19.09.00
Name and mailing address of the international preliminary examining authority:	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Heimann-Pohl, B Telephone No. +49 89 2399 8713

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/DK99/00382

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-25 as originally filed

Claims, No.:

1-27 as received on 13/07/2000 with letter of 10/07/2000

Drawings, sheets:

1/12-12/12 as originally filed

2. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-27
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-27
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-27
	No:	Claims	

2. Citations and explanations

see separate sheet

1). The present application relates to a method of preventing bacteriophage infection of bacterial cultures due to a mutation which renders the bacteria incapable of DNA replication, RNA transcription or protein synthesis, but which retain their capability of being metabolically active. The application further relates to the so defined bacteria and their use in the production of edible products.

2). Novelty, Inventive Step and Industrial Applicability (Box V)

2.1). Prior Art

D1: RICHARDSON G H ET AL: 'Proteinase negative variants of Streptococcus cremoris for cheese starters' JOURNAL OF DAIRY SCIENCE, vol. 66, 1983, pages 2278-2286, XP002082743 cited in the application
D2: NILSSON D & LAURIDSEN A A: 'Isolation of purine auxotrophic mutants of Lactococcus lactis and characterization of the gene hpt encoding hypoxanthine guanine phosphoribosyltransferase.' MOLECULAR AND GENERAL GENETICS, vol. 235, 1992, pages 359-364, XP002032525 cited in the application

D1 discloses proteinase negative variants of Streptococcus cremoris which are able to acidify milk. However, these variants are still growing, although at a lower rate than the proteinase positive strain, as stated on page 2278 "Generation times of proteinase negative cells were 3.5 times as long in cheese milk as those of proteinase positive, suggesting a probable reduction of bacteriophage and antibiotic problems."

D2 discloses the purine auxotrophic mutant DN105 of L. lactis (paragraph spanning page 360, right col.-page 361, left col.). D2 does not refer to the use of said mutant in food production.

2.2). Novelty

Growth, as in the case of D1, requires DNA replication, RNA transcription and protein synthesis, therefore variants of D1 do not appear to fall under the requirements set out in claim 1, 2, 3, 8-11 and 26. Also the subject matter of

claims 4-7, 12-25 and 27 appears to be novel with regard to D1.
The strains disclosed in D2 were not used in the methods of claims 1-17 and 24-27. Also the subject matter of claims 20-23 appears to be novel with regard to D2. The mutants DN101-104 which are also disclosed in D2 are disclaimed in claim 18 and 19, therefore said claims can be regarded as novel (Art. 33 (2) PCT).

2.3). Inventive Step

The problem underlying the present application is that bacterial cultures, used for example for the fermentation of milk, are often subject to bacteriophage infection. This problem is solved by the provision of bacteria that are purine or pyrimidine mutants but still capable of metabolizing substrate material.
It is common knowledge that DNA replication, RNA transcription and protein synthesis is necessary for bacteriophage development.
However, with regard to the description page 17, lines 11-13, it was apparently not known that purine auxotrophic mutants of L.lactis not growing in milk would be capable of acidifying such a substrate material.
Therefore the use of a purine auxotrophic mutant of L.lactis in the method of claims 1-4, 6-11and 27 involves an inventive step (overcoming of a prejudice).

The use of a pyrimidine (Pyr^-) mutant is not obviously derivable from the prior art. Therefore claims 5 and 20-27 are inventive.

In the method of claims 12-14 the strain not being susceptible to attack by bacteriophages due to not being capable of DNA replication, RNA transcription or protein synthesis is, additionally, genetically modified such that it has an enhanced metabolic activity relative to its parent strain. Although not reduced into practice in the present application, it seems likely that the skilled person would be able to obtain such a strain with regard to the teaching of example 2 (page 18 of the present application) in combination with page 11 and the teaching of WO 98/10089.
In the method of claims 15 and 16 the feature "not being capable of DNA replication, RNA transcription or protein synthesis" should be due to a conditional mutation. Although not reduced into practice in the present application, it seems nevertheless likely that the skilled person would be able to obtain such a strain

using his or her general knowledge of methods to use to provide such a conditional mutant.

In the method of claims 17 a strain which is not capable of DNA replication is unable to carry out mitosis, but it would still be able of growing in size, since, under this condition synthesis of new enzymes is not affected.

Thus also the methods of claims 12-17 can be considered to involve an inventive step.

2.4). Industrial Applicability

The subject matter of claims 1-27 appears to be industrial applicable.

21134PC1

Chr. Hansen A/S
International Patent Application No. PCT/DK99/00382
Publication No. WO 00/01799

AMENDED CLAIMS, July 2000

1. A method of modifying a substrate material by means of a bacterial culture which is capable of being metabolically active in said substrate, whereby the bacterial culture is not susceptible to attack by bacteriophages, the method comprising

(i) isolating a bacterial strain which is not capable of DNA replication, RNA transcription or protein synthesis in said substrate material but is capable of metabolically modifying the substrate material,

(ii) propagating the selected strain in a medium wherein the strain is capable of replicating to obtain a culture of said strain,

(iii) adding the thus obtained bacterial culture to the substrate material and keeping the material under conditions where the culture is metabolically active.

whereby, if the substrate material is contaminated with a bacteriophage, the metabolic activity of the bacterial culture is substantially unaffected by the bacteriophage.

2. A method according to claim 1 wherein the substrate material is limited with respect to at least one compound that is required by the bacterial strain for DNA replication, RNA transcription or protein synthesis.

3. A method according to claim 2 wherein the bacterial strain is a mutant strain being auxothrophic in respect of a compound which is not present in the substrate material and which is required by the strain for replication.

21134PC1

4. A method according to claim 3 wherein the mutant strain is a Pur⁻ mutant including *Lactococcus lactis* strain DN105 deposited under the accession number DSM 12289.

5. A method according to claim 3 wherein the mutant strain is a *thyA* mutant including *Lactococcus lactis* strain MBP71 deposited under the accession number DSM12891.

6. A method according to any of claims 2 to 5 wherein the strain in said substrate material is not capable of performing at least one activity selected from the group consisting of DNA replication, RNA transcription and protein synthesis.

7. A method according to claim 1 wherein the substrate material contains at least one compound that inhibits the DNA replication, RNA transcription or the protein synthesis of the bacterial strain.

8. A method according to claim 1 wherein the substrate material is a starting material for an edible product, the material is selected from the group consisting of milk, a vegetable material, a meat product, a must, a fruit juice, a wine, a dough and a batter.

9. A method according to claim 1 wherein the bacterial culture is selected from the group consisting of *Lactococcus* spp., *Lactobacillus* spp., *Leuconostoc* spp., *Pediococcus* spp., *Streptococcus* spp., *Propionibacterium* spp., *Bifidobacterium* spp., *Staphylococcus* spp., *Micrococcus* spp., *Bacillus* spp., *Enterobacteriaceae* spp. including *E. coli*, *Actinomycetes* spp., *Corynebacterium* spp. and *Brevibacterium* spp.

10. A method according to claim 9 wherein the bacterial culture is of *Lactococcus lactis*.

11. A method according to claim 1 wherein the bacterial strain is added to the substrate material at a concentration in the range of 10^5 to 10^9 CFU/ml or g of the material.

12. A method according to claim 1 wherein the culture comprises a genetically modified strain which, relative to its parent strain is enhanced in at least one metabolic pathway.

13. A method according to claim 12 wherein the genetically modified strain has, relative to its parent strain, an enhanced metabolic activity selected from the group consisting of enhanced glycolytic flux and enhanced flux through the pentose phosphate pathway.

14. A method according to claim 13 wherein the genetically modified strain has, relative to its parent strain, an enhanced ATPase activity.

15. A method according to claim 1 wherein the bacterial culture comprises a strain which is a conditional mutant which at a predetermined condition does not perform at least one activity selected from the group consisting of DNA replication, RNA transcription and protein synthesis.

16. A method according to claim 15 wherein the predetermined condition is selected from the group consisting of pH, temperature, composition of the substrate material and presence/absence of an inducer substance.

17. A method according to claim 1 wherein the culture comprises a bacterial strain which is capable of increasing the size of the cells without mitosis.

18. A modified lactic acid bacterium that is modified to become incapable of performing DNA replication, RNA transcription or protein synthesis in a specifically defined substrate material which is limited with respect to at least one compound that is required by the bacterial strain for DNA replication, RNA transcription or protein synthesis, said modified bacterial strain is capable of being metabolically active in said substrate material, whereby the strain is not susceptible to attack by bacteriophages, subject to the limitation, that the lactic acid bacterium is not a strain selected from the group consisting of strain DN101, DN102, DN103, DN104 and DN105 (DSM12289).

19. A lactic acid bacterium according to claim 18 wherein the bacterial strain is a mutant strain being auxothrophic in respect of a compound which is not present in the substrate material and which is required by the strain for replication.

20. A lactic acid bacterium according to claim 19 wherein the mutant strain is a *thyA* mutant including *Lactococcus lactis* strain MBP71 deposited under the accession number DSM12891.

21. A starter culture compostion comprising the lactic acid bacterium of any of claims 18-20.

22. A starter culture composition comprising a lactic acid bacterium obtainable by the method according to claim 1 in combination with at least one further lactic acid bacterium.

23. A composition according to claim 22 which further comprising at least on component enhancing the viability of the bacterial active ingrediant during storage including a bacterial nutrient or a cryoprotectant.

24. A method of manufacturing a food or feed product comprising adding a starter culture composition according to any of claims 21-23 to a food or feed product starting material and keeping the thus inoculated starting material under conditions where the lactic acid bacterium is metabolically active.

25. A method according to claim 24 wherein the food product starting material is milk.

26. Use of a culture as obtained in the method of claim 1 or a lactic acid bacterium according to any of claims 18-20 as a starter culture in the preparation of a product selected from the group consisting of a dairy flavour, a product for cheese flavouring, a food product and a feed product.

27. A method of preventing that a lactic acid bacterial starter culture is infected by bacteriophages in the manufacturing of a food or feed product, the method

21134PC1

comprising adding as a starter culture a lactic acid bacterium obtained by the method according to claim 1 to a food or feed product starting material which is limited with respect to at least one compound that is required by the bacterial strain for DNA replication, RNA transcription or protein synthesis and keeping the thus inoculated starting material under conditions where the lactic acid bacterium is metabolically active, whereby, if the substrate material is contaminated with a bacteriophage, the metabolic activity of the bacterial culture is substantially unaffected by the bacteriophage.

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PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
21134 PC 1		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/DK 99/ 00382	02/07/1999	03/07/1998
Applicant		
CHR. HANSEN A/S et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [X] contained in the international application in written form.

   [X] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. –

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
[redacted]/DK 99/00382

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N1/20 A23C19/032

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A23C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RICHARDSON G H ET AL: "Proteinase negative variants of Streptococcus cremoris for cheese starters" JOURNAL OF DAIRY SCIENCE, vol. 66, 1983, pages 2278-2286, XP002082743 cited in the application abstract page 2284, column 2 ---	1-3, 8-11,26
A	RICHARDSON G H ET AL: "Paired and single strain protease negative lactic Streptococci for cheese manufacture." JOURNAL OF DAIRY SCIENCE, vol. 67, no. 3, 1984, pages 518-521, XP002082744 cited in the application abstract --- -/--	1-3, 8-11,26

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 November 1999	11/11/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Lejeune, R

1

Form PCT/ISA/210 (second sheet) (July 1992)

International Application No
PCT/DK 99/00382

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	NILSSON D & LAURIDSEN A A: "Isolation of purine auxotrophic mutants of Lactococcus lactis and characterization of the gene hpt encoding hypoxanthine guanine phosphoribosyltransferase." MOLECULAR AND GENERAL GENETICS, vol. 235, 1992, pages 359-364, XP002032525 cited in the application abstract ---	4
A	SANDS J A & AUPERIN D: "Effects of temperature and host cell genetic characteristics on the replication of the lipid-containing bacteriophage PR4 in Escherichia coli." JOURNAL OF VIROLOGY, vol. 22, no. 2, 1977, pages 315-320, XP002082746 abstract ---	3
A	US 4 900 669 A (HATCH RANDOLPH T ET AL) 13 February 1990 (1990-02-13) the whole document ---	1
A	DATABASE WPI Section Ch, Week 8934 Derwent Publications Ltd., London, GB; Class B04, AN 89-246864 XP002082747 & SU 1 439 121 A (AS UKR MOLECULAR BIOL), 23 November 1988 (1988-11-23) abstract ---	1
A	WO 98 10089 A (JENSEN PETER RUHDAL ;SNOEP JACKY LEENDERT (NL); WESTERHOFF HANS VI) 12 March 1998 (1998-03-12) cited in the application abstract -----	13,14

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
/DK 99/00382

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 4900669 A	13-02-1990	US 4673640 A	16-06-1987
		AU 4179385 A	05-12-1985
		DK 191085 A	31-10-1985
		EP 0160571 A	06-11-1985
		FI 851682 A	31-10-1985
		JP 60244291 A	04-12-1985
SU 1439121 A	23-11-1988	NONE	
WO 9810089 A	12-03-1998	AU 4113597 A	26-03-1998
		EP 0939830 A	08-09-1999

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
21134 PC 1		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/DK 99/00382	02/07/1999	03/07/1998
Applicant		
CHR. HANSEN A/S et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

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   [X] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

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   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. –

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK 99/00382

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N1/20 C19/032

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A23C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RICHARDSON G H ET AL: "Proteinase negative variants of Streptococcus cremoris for cheese starters" JOURNAL OF DAIRY SCIENCE, vol. 66, 1983, pages 2278-2286, XP002082743 cited in the application abstract page 2284, column 2 ---	1-3, 8-11,26
A	RICHARDSON G H ET AL: "Paired and single strain protease negative lactic Streptococci for cheese manufacture." JOURNAL OF DAIRY SCIENCE, vol. 67, no. 3, 1984, pages 518-521, XP002082744 cited in the application abstract --- -/--	1-3, 8-11,26

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 November 1999	11/11/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Lejeune, R

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NILSSON D & LAURIDSEN A A: "Isolation of purine auxotrophic mutants of Lactococcus lactis and characterization of the gene hpt encoding hypoxanthine guanine phosphoribosyltransferase." MOLECULAR AND GENERAL GENETICS, vol. 235, 1992, pages 359-364, XP002032525 cited in the application abstract	4
A	SANDS J A & AUPERIN D: "Effects of temperature and host cell genetic characteristics on the replication of the lipid-containing bacteriophage PR4 in Escherichia coli." JOURNAL OF VIROLOGY, vol. 22, no. 2, 1977, pages 315-320, XP002082746 abstract	3
A	US 4 900 669 A (HATCH RANDOLPH T ET AL) 13 February 1990 (1990-02-13) the whole document	1
A	DATABASE WPI Section Ch, Week 8934 Derwent Publications Ltd., London, GB; Class B04, AN 89-246864 XP002082747 & SU 1 439 121 A (AS UKR MOLECULAR BIOL), 23 November 1988 (1988-11-23) abstract	1
A	WO 98 10089 A (JENSEN PETER RUHDAL ;SNOEP JACKY LEENDERT (NL); WESTERHOFF HANS VI) 12 March 1998 (1998-03-12) cited in the application abstract	13,14

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/DK 99/00382

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 4900669 A	13-02-1990	US 4673640 A	16-06-1987
		AU 4179385 A	05-12-1985
		DK 191085 A	31-10-1985
		EP 0160571 A	06-11-1985
		FI 851682 A	31-10-1985
		JP 60244291 A	04-12-1985
SU 1439121 A	23-11-1988	NONE	
WO 9810089 A	12-03-1998	AU 4113597 A	26-03-1998
		EP 0939830 A	08-09-1999

15

PATENT COOPERATION TREATY

REC'D 21 SEP 2000

WIPO PCT

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
21134 PC 1		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/DK99/00382	02/07/1999	03/07/1998
International Patent Classification (IPC) or national classification and IPC C12N1/20		
Applicant CHR. HANSEN A/S et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 5 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
17/12/1999	19.09.00
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Heimann-Pohl, B Telephone No. +49 89 2399 8713

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/DK99/00382

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-25 as originally filed

Claims, No.:

1-27 as received on 13/07/2000 with letter of 10/07/2000

Drawings, sheets:

1/12-12/12 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-27
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-27
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-27
	No:	Claims	

2. Citations and explanations

see separate sheet

1). The present application relates to a method of preventing bacteriophage infection of bacterial cultures due to a mutation which renders the bacteria incapable of DNA replication, RNA transcription or protein synthesis, but which retain their capability of being metabolically active. The application further relates to the so defined bacteria and their use in the production of edible products.

2). Novelty, Inventive Step and Industrial Applicability (Box V)

2.1). Prior Art

D1: RICHARDSON G H ET AL: 'Proteinase negative variants of Streptococcus cremoris for cheese starters' JOURNAL OF DAIRY SCIENCE, vol. 66, 1983, pages 2278-2286, XP002082743 cited in the application
D2: NILSSON D & LAURIDSEN A A: 'Isolation of purine auxotrophic mutants of Lactococcus lactis and characterization of the gene hpt encoding hypoxanthine guanine phosphoribosyltransferase.' MOLECULAR AND GENERAL GENETICS, vol. 235, 1992, pages 359-364, XP002032525 cited in the application

D1 discloses proteinase negative variants of Streptococcus cremoris which are able to acidify milk. However, these variants are still growing, although at a lower rate than the proteinase positive strain, as stated on page 2278 "Generation times of proteinase negative cells were 3.5 times as long in cheese milk as those of proteinase positive, suggesting a probable reduction of bacteriophage and antibiotic problems."

D2 discloses the purine auxotrophic mutant DN105 of L. lactis (paragraph spanning page 360, right col.-page 361, left col.). D2 does not refer to the use of said mutant in food production.

2.2). Novelty

Growth, as in the case of D1, requires DNA replication, RNA transcription and protein synthesis, therefore variants of D1 do not appear to fall under the requirements set out in claim 1, 2, 3, 8-11 and 26. Also the subject matter of

claims 4-7, 12-25 and 27 appears to be novel with regard to D1.
The strains disclosed in D2 were not used in the methods of claims 1-17 and 24-27. Also the subject matter of claims 20-23 appears to be novel with regard to D2.
The mutants DN101-104 which are also disclosed in D2 are disclaimed in claim 18 and 19, therefore said claims can be regarded as novel (Art. 33 (2) PCT).

2.3). Inventive Step

The problem underlying the present application is that bacterial cultures, used for example for the fermentation of milk, are often subject to bacteriophage infection. This problem is solved by the provision of bacteria that are purine or pyrimidine mutants but still capable of metabolizing substrate material.
It is common knowledge that DNA replication, RNA transcription and protein synthesis is necessary for bacteriophage development.
However, with regard to the description page 17, lines 11-13, it was apparently not known that purine auxotrophic mutants of L.lactis not growing in milk would be capable of acidifying such a substrate material.
Therefore the use of a purine auxotrophic mutant of L.lactis in the method of claims 1-4, 6-11and 27 involves an inventive step (overcoming of a prejudice).

The use of a pyrimidine (Pyr$^-$) mutant is not obviously derivable from the prior art. Therefore claims 5 and 20-27 are inventive.

In the method of claims 12-14 the strain not being susceptible to attack by bacteriophages due to not being capable of DNA replication, RNA transcription or protein synthesis is, additionally, genetically modified such that it has an enhanced metabolic activity relative to its parent strain. Although not reduced into practice in the present application, it seems likely that the skilled person would be able to obtain such a strain with regard to the teaching of example 2 (page 18 of the present application) in combination with page 11 and the teaching of WO 98/10089.
In the method of claims 15 and 16 the feature "not being capable of DNA replication, RNA transcription or protein synthesis" should be due to a conditional mutation. Although not reduced into practice in the present application, it seems nevertheless likely that the skilled person would be able to obtain such a strain

using his or her general knowledge of methods to use to provide such a conditional mutant.

In the method of claims 17 a strain which is not capable of DNA replication is unable to carry out mitosis, but it would still be able of growing in size, since, under this condition synthesis of new enzymes is not affected.

Thus also the methods of claims 12-17 can be considered to involve an inventive step.

2.4). Industrial Applicability

The subject matter of claims 1-27 appears to be industrial applicable.

CLAIMS

1. A method of modifying a substrate material by means of a bacterial culture which is capable of being metabolically active in said substrate, whereby the bacterial culture is not susceptible to attack by bacteriophages, the method comprising

(i) isolating a bacterial strain which is not capable of DNA replication, RNA transcription or protein synthesis in said substrate material but is capable of metabolically modifying the substrate material,

(ii) propagating the selected strain in a medium wherein the strain is capable of replicating to obtain a culture of said strain,

(iii) adding the thus obtained bacterial culture to the substrate material and keeping the material under conditions where the culture is metabolically active.

whereby, if the substrate material is contaminated with a bacteriophage, the metabolic activity of the bacterial culture is substantially unaffected by the bacteriophage.

2. A method according to claim 1 wherein the substrate material is limited with respect to at least one compound that is required by the bacterial strain for DNA replication, RNA transcription or protein synthesis.

3. A method according to claim 2 wherein the bacterial strain is a mutant strain being auxothrophic in respect of a compound which is not present in the substrate material and which is required by the strain for replication.

4. A method according to claim 3 wherein the mutant strain is a Pur$^-$ mutant including *Lactococcus lactis* strain DN105 deposited under the accession number DSM 12289.

5. A method according to claim 3 wherein the mutant strain is a *thyA* mutant including *Lactococcus lactis* strain MBP71 deposited under the accession number DSM12891.

6. A method according to any of claims 2 to 5 wherein the strain in said substrate material is not capable of performing at least one activity selected from the group consisting of DNA replication, RNA transcription and protein synthesis.

7. A method according to claim 1 wherein the substrate material contains at least one compound that inhibits the DNA replication, RNA transcription or the protein synthesis of the bacterial strain.

8. A method according to claim 1 wherein the substrate material is a starting material for an edible product, the material is selected from the group consisting of milk, a vegetable material, a meat product, a must, a fruit juice, a wine, a dough and a batter.

9. A method according to claim 1 wherein the bacterial culture is selected from the group consisting of *Lactococcus* spp., *Lactobacillus* spp., *Leuconostoc* spp., *Pediococcus* spp., *Streptococcus* spp., *Propionibacterium* spp., *Bifidobacterium* spp., *Staphylococcus* spp., *Micrococcus* spp., *Bacillus* spp., *Enterobacteriaceae* spp. including *E. coli*, *Actinomycetes* spp., *Corynebacterium* spp. and *Brevibacterium* spp.

10. A method according to claim 9 wherein the bacterial culture is of *Lactococcus lactis*.

11. A method according to claim 1 wherein the bacterial strain is added to the substrate material at a concentration in the range of 10^5 to 10^9 CFU/ml or g of the material.

12. A method according to claim 1 wherein the culture comprises a genetically modified strain which, relative to its parent strain is enhanced in at least one metabolic pathway.

13. A method according to claim 12 wherein the genetically modified strain has, relative to its parent strain, an enhanced metabolic activity selected from the group consisting of enhanced glycolytic flux and enhanced flux through the pentose phosphate pathway.

14. A method according to claim 13 wherein the genetically modified strain has, relative to its parent strain, an enhanced ATPase activity.

15. A method according to claim 1 wherein the bacterial culture comprises a strain which is a conditional mutant which at a predetermined condition does not perform at least one activity selected from the group consisting of DNA replication, RNA transcription and protein synthesis.

16. A method according to claim 15 wherein the predetermined condition is selected from the group consisting of pH, temperature, composition of the substrate material and presence/absence of an inducer substance.

17. A method according to claim 1 wherein the culture comprises a bacterial strain which is capable of increasing the size of the cells without mitosis.

18. A modified lactic acid bacterium that is modified to become incapable of performing DNA replication, RNA transcription or protein synthesis in a specifically defined substrate material which is limited with respect to at least one compound that is required by the bacterial strain for DNA replication, RNA transcription or protein synthesis, said modified bacterial strain is capable of being metabolically active in said substrate material, whereby the strain is not susceptible to attack by bacteriophages, subject to the limitation, that the lactic acid bacterium is not strain DN105 (DSM12289).

19. A lactic acid bacterium according to claim 18 wherein the bacterial strain is a mutant strain being auxothrophic in respect of a compound which is not present in the substrate material and which is required by the strain for replication.

20. A lactic acid bacterium according to claim 19 wherein the mutant strain is a *thyA* mutant including *Lactococcus lactis* strain MBP71 deposited under the accession number DSM12891.

21. A starter culture compostion comprising the lactic acid bacterium of any of claims 18-20.

22. A starter culture composition comprising a lactic acid bacterium obtainable by the method according to claim 1 in combination with at least one further lactic acid bacterium.

23. A composition according to claim 22 which further comprising at least on component enhancing the viability of the bacterial active ingredient during storage including a bacterial nutrient or a cryoprotectant.

24. A method of manufacturing a food or feed product comprising adding a starter culture composition according to any of claims 21-23 to a food or feed product starting material and keeping the thus inoculated starting material under conditions where the lactic acid bacterium is metabolically active.

25. A method according to claim 24 wherein the food product starting material is milk.

26. Use of a culture as obtained in the method of claim 1 or a lactic acid bacterium according to any of claims 18-20 as a starter culture in the preparation of a product selected from the group consisting of a dairy flavour, a product for cheese flavouring, a food product and a feed product.

27. A method of preventing that a lactic acid bacterial starter culture is infected by bacteriophages in the manufacturing of a food or feed product, the method comprising adding as a starter culture a lactic acid bacterium obtained by the method according to claim 1 to a food or feed product starting material which is limited with respect to at least one compound that is required by the bacterial strain for DNA replication, RNA transcription or protein synthesis and keeping the thus inoculated starting material under conditions where the lactic acid bacterium is metabolically active, whereby, if the substrate material is contaminated with a bacteriophage, the metabolic activity of the bacterial culture is substantially unaffected by the bacteriophage.

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WO 00/01799 A3

(54) Title: METHOD OF PREVENTING BACTERIOPHAGE INFECTION OF BACTERIAL CULTURES

(57) Abstract: Method of preventing bacteriophage infection of bacterial cultures comprising modified strains, wherein the cultures are completely resistant to bacteriophage attack and have retained their capability of being metabolically active. The method is useful in the manufacturing of food products, feed products or useful metabolite products.

INTERNATIONAL SEARCH REPORT

Inter tional Application No
PCI/DK 99/00382

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N1/20 A23C19/032

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A23C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RICHARDSON G H ET AL: "Proteinase negative variants of Streptococcus cremoris for cheese starters" JOURNAL OF DAIRY SCIENCE, vol. 66, 1983, pages 2278-2286, XP002082743 cited in the application abstract page 2284, column 2 ---	1-3, 8-11,26
A	RICHARDSON G H ET AL: "Paired and single strain protease negative lactic Streptococci for cheese manufacture." JOURNAL OF DAIRY SCIENCE, vol. 67, no. 3, 1984, pages 518-521, XP002082744 cited in the application abstract --- -/--	1-3, 8-11,26

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
4 November 1999	11/11/1999

Name and mailing address of the ISA	Authorized officer
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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter 'tional Application No
PCI/DK 99/00382

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	NILSSON D & LAURIDSEN A A: "Isolation of purine auxotrophic mutants of Lactococcus lactis and characterization of the gene hpt encoding hypoxanthine guanine phosphoribosyltransferase." MOLECULAR AND GENERAL GENETICS, vol. 235, 1992, pages 359-364, XP002032525 cited in the application abstract	4
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A	DATABASE WPI Section Ch, Week 8934 Derwent Publications Ltd., London, GB; Class B04, AN 89-246864 XP002082747 & SU 1 439 121 A (AS UKR MOLECULAR BIOL), 23 November 1988 (1988-11-23) abstract	1
A	WO 98 10089 A (JENSEN PETER RUHDAL ;SNOEP JACKY LEENDERT (NL); WESTERHOFF HANS VI) 12 March 1998 (1998-03-12) cited in the application abstract	13,14

INTERNATIONAL SEARCH REPORT

nformation on patent family members

Inte tional Application No
PCI/DK 99/00382

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 4900669 A	13-02-1990	US 4673640 A	16-06-1987
		AU 4179385 A	05-12-1985
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WO 9810089 A	12-03-1998	AU 4113597 A	26-03-1998
		EP 0939830 A	08-09-1999

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Our ref: 21134 PC 1

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Dear Sirs,

In response to your first Written Opinion, dated 20 April 2000, we hereby submit an amended set of claims and provide the below arguments.

For your convenience we enclose a set of the previous claims with our amendments in handwriting.

Claim 18 has been amended by inserting the expression "is not a strain selected from the group consisting of strain DN101, DN102, DN103 and DN104" before "DN105 (DSM12289)".

By amending the claim in this manner, the Applicant trusts that the novelty objections raised in the Written Opinion have been duly overcome.

The IPEA states, that it considers the features of claims 12-17 to be not sufficiently disclosed in the application, since it has not been shown that the genetically modified strain indeed solves the problem of not being susceptible to attack of bacteriophages and that these strains have an enhanced metabolic activity relative to its parent strain.

With regard to this objection the Applicants submits the following arguments:

Regarding claims 12 to 14

As described in the present specification, the method of the present invention provides cells resistant to bacteriophage attacks through abolishment of DNA replication, RNA transcription, or protein synthesis under certain conditions. As an example DNA replication can be made dependent on the presence of thymidine in the growth medium through the inactivation of the thyA (thymidylate synthase)

gene (Example 2, page 18). As explained in the present specification, biomass of such a mutant can be produced by adding thymidine to an appropriate growth medium. Under these conditions DNA replication functions as normal. However, when biomass of such a strain is added to a substrate material devoid of thymidine, such as milk, DNA replication is no longer possible. Nonetheless, RNA transcription and protein synthesis are not directly affected and will still function. Specifically, since each cell contain at least one chromosome, genes can be transcribed into mRNA and proteins can be synthesised. Since the cell number can not increase for such a thyA mutant one would suspect such a culture to be less metabolically active than an exponentially growing wild-type culture.

Thus, as explained on page 11, line11 to page 12, line 32 a mutant useful in the present invention may be further developed through enhancing one or more of the metabolic pathways, i.e. increasing the metabolic activity. The description is very detailed with respect to how to achieve this enhancement, i.e. by increasing the expression of ATPase activity as disclosed in WO 98/10089. Therefore, no undue experimentation is required to generate strains that have en enhanced metabolic activity due to e.g. an enhanced ATPase activity as claimed in claim 14.

The ATPase will hydrolyse ATP and drive the energy generating reactions, i.e. glycolysis in *L. lactis* to a higher rate, whereby lactic acid is produced faster. The genes employed to enhance a metabolic pathway, would normally be located on a plasmid, but could also be integrated in the chromosome. This plasmid would be replicated similarly to the chromosome when biomass of the culture is produced. When this type of enhanced strain is added to e.g. milk the plasmid can no longer be replicated. However, the plasmids which are already present inside the cells will persist and the relevant genes can be expressed, since the functions of RNA replication and protein synthesis are active. Even if RNA replication and protein synthesis were not active in a certain bacteriophage resistant strain, the relevant enzymes could already be present inside the cell from when the biomass was produced. Because this type of strain is bacteriophage resistant due to the lack of DNA replication, RNA replication, or protein synthesis, the enhancement of one or more metabolic pathways does not affect the bacteriophage resistance. In conclusion, a person of ordinary skilled in the art can easily appreciate that the enhancement of one or more metabolic pathways would not interfere with capability to resist attack by bacteriophages and thus there is no reason to believe that the genetically modified strains useful in the method claimed in claims 12-14 would not solve the problem of the present invention, i.e. not being susceptible to attack by bacteriophages.

Regarding claims 15 and16

In the method claimed in claims 15 and 16 the bacterial culture comprises a bacterial strain which is a conditional mutant. It is known in the art that a conditional mutant strain having a specific enzyme which is active at e.g. 30°C, but not 37°C, can be provided by altering the amino acid sequence of the enzyme. If this specific enzyme is e.g. thymidylate synthase, encoded by thyA, it is easy to see how the culture would be completely bacteriophage resistant at the higher temperature of 37°C, but not at 30°C. Thus, no undue experimentation is required to generate a

conditional mutant which at a predetermined condition, i.e. a specific temperature or pH, does not perform DNA replication, RNA transcription or protein synthesis, since a person skilled in the art would know which methods to use to provide such a conditional mutant.

Regarding claim 17

It is well known in the art that a strain which is not capable of DNA replication, RNA transcription, or protein synthesis will not be able to carry out mitosis. If DNA replication is not possible, the chromosome can not be replicated, and mitosis is not possible. If RNA transcription or protein synthesis are not possible the relevant enzymes required to mitosis can not be synthesised. In any case the cells can not carry out mitosis, but they will be completely bacteriophage resistant. If it is DNA replication which is abolished the cells will still be able to synthesise new enzymes and hence capable of growing in size, in the same manner as cells normally increase in size before mitosis.

In case that IPEA finds that not all of the objections raised in the first Written Opinion have been properly repudiated by this reply, or if the IPEA objects to any of the new claims as submitted with this response, Applicants kindly requests that at least one additional opportunity to submit amendments and arguments be given (Rule 66.4b, PCT) before the issuance of the Preliminary Examination Report.

Yours sincerely,

Plougmann, Vingtoft & Partners

Hep

Heidi Petersen

Amended claims
Originally filed claims with amendments in handwriting
Form 1037

Chr. Hansen A/S
International Patent Application No. PCT/DK99/00382
Publication No. WO 00/01799

AMENDED CLAIMS, July 2000

1. A method of modifying a substrate material by means of a bacterial culture which is capable of being metabolically active in said substrate, whereby the bacterial culture is not susceptible to attack by bacteriophages, the method comprising

(i) isolating a bacterial strain which is not capable of DNA replication, RNA transcription or protein synthesis in said substrate material but is capable of metabolically modifying the substrate material,

(ii) propagating the selected strain in a medium wherein the strain is capable of replicating to obtain a culture of said strain,

(iii) adding the thus obtained bacterial culture to the substrate material and keeping the material under conditions where the culture is metabolically active.

whereby, if the substrate material is contaminated with a bacteriophage, the metabolic activity of the bacterial culture is substantially unaffected by the bacteriophage.

2. A method according to claim 1 wherein the substrate material is limited with respect to at least one compound that is required by the bacterial strain for DNA replication, RNA transcription or protein synthesis.

3. A method according to claim 2 wherein the bacterial strain is a mutant strain being auxothrophic in respect of a compound which is not present in the substrate material and which is required by the strain for replication.

4. A method according to claim 3 wherein the mutant strain is a Pur⁻ mutant including *Lactococcus lactis* strain DN105 deposited under the accession number DSM 12289.

5. A method according to claim 3 wherein the mutant strain is a *thyA* mutant including *Lactococcus lactis* strain MBP71 deposited under the accession number DSM12891.

6. A method according to any of claims 2 to 5 wherein the strain in said substrate material is not capable of performing at least one activity selected from the group consisting of DNA replication, RNA transcription and protein synthesis.

7. A method according to claim 1 wherein the substrate material contains at least one compound that inhibits the DNA replication, RNA transcription or the protein synthesis of the bacterial strain.

8. A method according to claim 1 wherein the substrate material is a starting material for an edible product, the material is selected from the group consisting of milk, a vegetable material, a meat product, a must, a fruit juice, a wine, a dough and a batter.

9. A method according to claim 1 wherein the bacterial culture is selected from the group consisting of *Lactococcus* spp., *Lactobacillus* spp., *Leuconostoc* spp., *Pediococcus* spp., *Streptococcus* spp., *Propionibacterium* spp., *Bifidobacterium* spp., *Staphylococcus* spp., *Micrococcus* spp., *Bacillus* spp., *Enterobacteriaceae* spp. including *E. coli*, *Actinomycetes* spp., *Corynebacterium* spp. and *Brevibacterium* spp.

10. A method according to claim 9 wherein the bacterial culture is of *Lactococcus lactis*.

11. A method according to claim 1 wherein the bacterial strain is added to the substrate material at a concentration in the range of 10^5 to 10^9 CFU/ml or g of the material.

12. A method according to claim 1 wherein the culture comprises a genetically modified strain which, relative to its parent strain is enhanced in at least one metabolic pathway.

13. A method according to claim 12 wherein the genetically modified strain has, relative to its parent strain, an enhanced metabolic activity selected from the group consisting of enhanced glycolytic flux and enhanced flux through the pentose phosphate pathway.

14. A method according to claim 13 wherein the genetically modified strain has, relative to its parent strain, an enhanced ATPase activity.

15. A method according to claim 1 wherein the bacterial culture comprises a strain which is a conditional mutant which at a predetermined condition does not perform at least one activity selected from the group consisting of DNA replication, RNA transcription and protein synthesis.

16. A method according to claim 15 wherein the predetermined condition is selected from the group consisting of pH, temperature, composition of the substrate material and presence/absence of an inducer substance.

17. A method according to claim 1 wherein the culture comprises a bacterial strain which is capable of increasing the size of the cells without mitosis.

18. A modified lactic acid bacterium that is modified to become incapable of performing DNA replication, RNA transcription or protein synthesis in a specifically defined substrate material which is limited with respect to at least one compound that is required by the bacterial strain for DNA replication, RNA transcription or protein synthesis, said modified bacterial strain is capable of being metabolically active in said substrate material, whereby the strain is not susceptible to attack by bacteriophages, subject to the limitation, that the lactic acid bacterium is not a strain selected from the group consisting of strain DN101, DN102, DN103, DN104 and DN105 (DSM12289).

19. A lactic acid bacterium according to claim 18 wherein the bacterial strain is a mutant strain being auxothrophic in respect of a compound which is not present in the substrate material and which is required by the strain for replication.

20. A lactic acid bacterium according to claim 19 wherein the mutant strain is a *thyA* mutant including *Lactococcus lactis* strain MBP71 deposited under the accession number DSM12891.

21. A starter culture compostion comprising the lactic acid bacterium of any of claims 18-20.

22. A starter culture composition comprising a lactic acid bacterium obtainable by the method according to claim 1 in combination with at least one further lactic acid bacterium.

23. A composition according to claim 22 which further comprising at least on component enhancing the viability of the bacterial active ingredient during storage including a bacterial nutrient or a cryoprotectant.

24. A method of manufacturing a food or feed product comprising adding a starter culture composition according to any of claims 21-23 to a food or feed product starting material and keeping the thus inoculated starting material under conditions where the lactic acid bacterium is metabolically active.

25. A method according to claim 24 wherein the food product starting material is milk.

26. Use of a culture as obtained in the method of claim 1 or a lactic acid bacterium according to any of claims 18-20 as a starter culture in the preparation of a product selected from the group consisting of a dairy flavour, a product for cheese flavouring, a food product and a feed product.

27. A method of preventing that a lactic acid bacterial starter culture is infected by bacteriophages in the manufacturing of a food or feed product, the method

comprising adding as a starter culture a lactic acid bacterium obtained by the method according to claim 1 to a food or feed product starting material which is limited with respect to at least one compound that is required by the bacterial strain for DNA replication, RNA transcription or protein synthesis and keeping the thus inoculated starting material under conditions where the lactic acid bacterium is metabolically active, whereby, if the substrate material is contaminated with a bacteriophage, the metabolic activity of the bacterial culture is substantially unaffected by the bacteriophage.

Chr. Hansen A/S
International Patent Application No. PCT/DK99/00382
Publication No. WO 00/01799

CLAIMS as filed

1. A method of modifying a substrate material by means of a bacterial culture which is capable of being metabolically active in said substrate, whereby the bacterial culture is not susceptible to attack by bacteriophages, the method comprising

(i) isolating a bacterial strain which is not capable of DNA replication, RNA transcription or protein synthesis in said substrate material but is capable of metabolically modifying the substrate material,

(ii) propagating the selected strain in a medium wherein the strain is capable of replicating to obtain a culture of said strain,

(iii) adding the thus obtained bacterial culture to the substrate material and keeping the material under conditions where the culture is metabolically active.

whereby, if the substrate material is contaminated with a bacteriophage, the metabolic activity of the bacterial culture is substantially unaffected by the bacteriophage.

2. A method according to claim 1 wherein the substrate material is limited with respect to at least one compound that is required by the bacterial strain for DNA replication, RNA transcription or protein synthesis.

3. A method according to claim 2 wherein the bacterial strain is a mutant strain being auxothrophic in respect of a compound which is not present in the substrate material and which is required by the strain for replication.

4. A method according to claim 3 wherein the mutant strain is a Pur$^-$ mutant including *Lactococcus lactis* strain DN105 deposited under the accession number DSM 12289.

5. A method according to claim 3 wherein the mutant strain is a *thyA* mutant including *Lactococcus lactis* strain MBP71 deposited under the accession number DSM12891.

6. A method according to any of claims 2 to 5 wherein the strain in said substrate material is not capable of performing at least one activity selected from the group consisting of DNA replication, RNA transcription and protein synthesis.

7. A method according to claim 1 wherein the substrate material contains at least one compound that inhibits the DNA replication, RNA transcription or the protein synthesis of the bacterial strain.

8. A method according to claim 1 wherein the substrate material is a starting material for an edible product, the material is selected from the group consisting of milk, a vegetable material, a meat product, a must, a fruit juice, a wine, a dough and a batter.

9. A method according to claim 1 wherein the bacterial culture is selected from the group consisting of *Lactococcus* spp., *Lactobacillus* spp., *Leuconostoc* spp., *Pediococcus* spp., *Streptococcus* spp., *Propionibacterium* spp., *Bifidobacterium* spp., *Staphylococcus* spp., *Micrococcus* spp., *Bacillus* spp., *Enterobacteriaceae* spp. including *E. coli*, *Actinomycetes* spp., *Corynebacterium* spp. and *Brevibacterium* spp.

10. A method according to claim 9 wherein the bacterial culture is of *Lactococcus lactis*.

11. A method according to claim 1 wherein the bacterial strain is added to the substrate material at a concentration in the range of 10^5 to 10^9 CFU/ml or g of the material.

12. A method according to claim 1 wherein the culture comprises a genetically modified strain which, relative to its parent strain is enhanced in at least one metabolic pathway.

13. A method according to claim 12 wherein the genetically modified strain has, relative to its parent strain, an enhanced metabolic activity selected from the group consisting of enhanced glycolytic flux and enhanced flux through the pentose phosphate pathway.

14. A method according to claim 13 wherein the genetically modified strain has, relative to its parent strain, an enhanced ATPase activity.

15. A method according to claim 1 wherein the bacterial culture comprises a strain which is a conditional mutant which at a predetermined condition does not perform at least one activity selected from the group consisting of DNA replication, RNA transcription and protein synthesis.

16. A method according to claim 15 wherein the predetermined condition is selected from the group consisting of pH, temperature, composition of the substrate material and presence/absence of an inducer substance.

17. A method according to claim 1 wherein the culture comprises a bacterial strain which is capable of increasing the size of the cells without mitosis.

18. A modified lactic acid bacterium that is modified to become incapable of performing DNA replication, RNA transcription or protein synthesis in a specifically defined substrate material which is limited with respect to at least one compound that is required by the bacterial strain for DNA replication, RNA transcription or protein synthesis, said modified bacterial strain is capable of being metabolically active in said substrate material, whereby the strain is not susceptible to attack by bacteriophages, subject to the limitation, that the lactic acid bacterium is not strain DN105 (DSM12289).

(a) selected from the group consiting of strain DN 101, DN 102, DN 103, DN 104 and

19. A lactic acid bacterium according to claim 18 wherein the bacterial strain is a mutant strain being auxothrophic in respect of a compound which is not present in the substrate material and which is required by the strain for replication.

20. A lactic acid bacterium according to claim 19 wherein the mutant strain is a *thyA* mutant including *Lactococcus lactis* strain MBP71 deposited under the accession number DSM12891.

21. A starter culture compostion comprising the lactic acid bacterium of any of claims 18-20.

22. A starter culture composition comprising a lactic acid bacterium obtainable by the method according to claim 1 in combination with at least one further lactic acid bacterium.

23. A composition according to claim 22 which further comprising at least on component enhancing the viability of the bacterial active ingredient during storage including a bacterial nutrient or a cryoprotectant.

24. A method of manufacturing a food or feed product comprising adding a starter culture composition according to any of claims 21-23 to a food or feed product starting material and keeping the thus inoculated starting material under conditions where the lactic acid bacterium is metabolically active.

25. A method according to claim 24 wherein the food product starting material is milk.

26. Use of a culture as obtained in the method of claim 1 or a lactic acid bacterium according to any of claims 18-20 as a starter culture in the preparation of a product selected from the group consisting of a dairy flavour, a product for cheese flavouring, a food product and a feed product.

27. A method of preventing that a lactic acid bacterial starter culture is infected by bacteriophages in the manufacturing of a food or feed product, the method

comprising adding as a starter culture a lactic acid bacterium obtained by the method according to claim 1 to a food or feed product starting material which is limited with respect to at least one compound that is required by the bacterial strain for DNA replication, RNA transcription or protein synthesis and keeping the thus inoculated starting material under conditions where the lactic acid bacterium is metabolically active, whereby, if the substrate material is contaminated with a bacteriophage, the metabolic activity of the bacterial culture is substantially unaffected by the bacteriophage.

The demand must be filed directly with the competent International Preliminary Examining Authority or, if two or more Authorities are competent, with the one chosen by the applicant. The full name or two-letter code of that Authority may be indicated by the applicant on the line below:

IPEA/ EPO

PCT

CHAPTER II

DEMAND

under Article 31 of the Patent Cooperation Treaty:
The undersigned requests that the international application specified below be the subject of international preliminary examination according to the Patent Cooperation Treaty and hereby elects all eligible States (except where otherwise indicated).

For International Preliminary Examining Authority use only

Identification of IPEA	Date of receipt of DEMAND

Box No. I IDENTIFICATION OF THE INTERNATIONAL APPLICATION

Applicant's or agent's file reference: 21134 PC 1

International application No.	International filing date *(day/month/year)*	(Earliest) Priority date *(day/month/year)*
PCT/DK99/00382	2 July 1999	3 July 1998

Title of invention: Method of preventing bacteriophage infection of bacterial cultures

Box No. II APPLICANT(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

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☐ Further applicants are indicated on a continuation sheet.

International application No.
PCT/DK99/00382

Box No. III AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The following person is [X] agent [ ] common representative

and [X] has been appointed earlier and represents the applicant(s) also for international preliminary examination.

[ ] is hereby appointed and any earlier appointment of (an) agent(s)/common representative is hereby revoked.

[ ] is hereby appointed, specifically for the procedure before the International Preliminary Examining Authority, in addition to the agent(s)/common representative appointed earlier.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

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Teleprinter No.:

[ ] **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Box No. IV BASIS FOR INTERNATIONAL PRELIMINARY EXAMINATION

Statement concerning amendments:*

1. The applicant wishes the international preliminary examination **to start on the basis of:**

[X] the international application as originally filed

the description [X] as originally filed
[ ] as amended under Article 34

the claims [X] as originally filed
[ ] as amended under Article 19 (together with any accompanying statement)
[ ] as amended under Article 34

the drawings [X] as originally filed
[ ] as amended under Article 34

2. [ ] The applicant wishes any amendment to the claims under Article 19 to be considered as reversed.

3. [ ] The applicant wishes the start of the international preliminary examination **to be postponed** until the expiration of 20 months from the priority date unless the International Preliminary Examining Authority receives a copy of any amendments made under Article 19 or a notice from the applicant that he does not wish to make such amendments (Rule 69.1(d)). *(This check-box may be marked only where the time limit under Article 19 has not yet expired.)*

* Where no check-box is marked, international preliminary examination will start on the basis of the international application as originally filed or, where a copy of amendments to the claims under Article 19 and/or amendments of the international application under Article 34 are received by the International Preliminary Examining Authority before it has begun to draw up a written opinion or the international preliminary examination report, as so amended.

Language for the purposes of international preliminary examination: English

[X] which is the language in which the international application was filed.
[ ] which is the language of a translation furnished for the purposes of international search.
[ ] which is the language of publication of the international application.
[ ] which is the language of the translation (to be) furnished for the purposes of international preliminary examination.

Box No. V ELECTION OF STATES

The applicant hereby **elects all eligible States** *(that is, all States which have been designated and which are bound by Chapter II of the PCT)*

excluding the following States which the applicant wishes **not to elect:**

International application No.
PCT/DK99/00382

Box No. VI CHECK LIST

The demand is accompanied by the following elements, in the language referred to in Box No. IV, for the purposes of international preliminary examination:			For International Preliminary Examining Authority use only: received	For International Preliminary Examining Authority use only: not received
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2. amendments under Article 34	:	sheets	☐	☐
3. copy (or, where required, translation) of amendments under Article 19	:	sheets	☐	☐
4. copy (or, where required, translation) of statement under Article 19	:	sheets	☐	☐
5. letter	: 1	sheets	☐	☐
6. other *(specify)*	:	sheets	☐	☐

The demand is also accompanied by the item(s) marked below:

1. [x] fee calculation sheet
2. [ ] separate signed power of attorney
3. [ ] copy of general power of attorney; reference number, if any:
4. [ ] statement explaining lack of signature
5. [ ] nucleotide and or amino acid sequence listing in computer readable form
6. [ ] other *(specify)*:

Box No. VII SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the demand).

Plougmann, Vingtoft & Partners A/S
Copenhagen, 16 December 1999

Hep
Heidi Petersen

For International Preliminary Examining Authority use only

1. Date of actual receipt of DEMAND:
2. Adjusted date of receipt of demand due to CORRECTIONS under Rule 60.1(b):
3. [ ] The date of receipt of the demand is AFTER the expiration of 19 months from the priority date and item 4 or 5, below, does not apply. [ ] The applicant has been informed accordingly.
4. [ ] The date of receipt of the demand is WITHIN the period of 19 months from the priority date as extended by virtue of Rule 80.5.
5. [ ] Although the date of receipt of the demand is after the expiration of 19 months from the priority date, the delay in arrival is EXCUSED pursuant to Rule 82.

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Demand received from IPEA on:

PATENT COOPERATION TREATY

From the:
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:
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DANEMARK

PLOUGMANN VINGTOFT & PARTNERS
25 APR. 2000
HEP/LPJ

PCT

WRITTEN OPINION

(PCT Rule 66)

Date of mailing *(day/month/year)*	20.04.2000
Applicant's or agent's file reference: 21134 PC.1	**REPLY DUE** **within 3 month(s)** from the above date of mailing

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/DK99/00382	02/07/1999	03/07/1998

International Patent Classification (IPC) or both national classification and IPC
C12N1/20

Applicant
CHR. HANSEN A/S et al.

1. This written opinion is the **first** drawn up by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

I	☒	Basis of the opinion
II	☐	Priority
III	☐	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Lack of unity of invention
V	☒	Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☐	Certain document cited
VII	☐	Certain defects in the international application
VIII	☐	Certain observations on the international application

3. The applicant is hereby **invited to reply** to this opinion.

When? See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also: For an additional opportunity to submit amendments, see Rule 66.4.
For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 bis.
For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 03/11/2000.

Name and mailing address of the international preliminary examining authority:
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Fax: +49 89 2399 - 4465

Authorized officer / Examiner
Heimann-Pohl, B

Formalities officer (incl. extension of time limits)
Vullo, C
Telephone No. +49 89 2399 8061

I. Basis of the opinion

1. This opinion has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed".*):

Description, pages:

1-25 as originally filed

Claims, No.:

1-27 as originally filed

Drawings, sheets:

1/12-12/12 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. This opinion has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	18,19
Inventive step (IS)	Claims	12-17
Industrial applicability (IA)	Claims	

2. Citations and explanations

see separate sheet

1). The present application relates to a method of preventing bacteriophage infection of bacterial cultures due to a mutation which renders the bacteria incapable of DNA replication, RNA transcription or protein synthesis, but which retain their capability of being metabolically active. The application further relates to the so defined bacteria and their use in the production of edible products.

2). Prior Art

D1: RICHARDSON G H ET AL: 'Proteinase negative variants of Streptococcus cremoris for cheese starters' JOURNAL OF DAIRY SCIENCE, vol. 66, 1983, pages 2278-2286, XP002082743 cited in the application

D2: NILSSON D & LAURIDSEN A A: 'Isolation of purine auxotrophic mutants of Lactococcus lactis and characterization of the gene hpt encoding hypoxanthine guanine phosphoribosyltransferase.' MOLECULAR AND GENERAL GENETICS, vol. 235, 1992, pages 359-364, XP002032525 cited in the application

D1 discloses proteinase negative variants of Streptococcus cremoris which are able to acidify milk. However, these variants are still growing, although at a lower rate than the proteinase positive strain, as stated on page 2278 "Generation times of proteinase negative cells were 3.5 times as long in cheese milk as those of proteinase positive, suggesting a probable reduction of bacteriophage and antibiotic problems."

D2 discloses the purine auxotrophic mutant DN105 of L. lactis (paragraph spanning page 360, right col.-page 361, left col.). D2 does not refer to the use of said mutant in food production.

3). Novelty

Growth, as in the case of D1, requires DNA replication, RNA transcription and protein synthesis, therefore variants of D1 do not appear to fall under the requirements set out in claim 1, 2, 3, 8-11 and 26. Also the subject matter of claims 4-7, 12-25 and 27 appears to be novel with regard to D1.

The strains disclosed in D2 were not used in the methods of claims 1-17 and 24-27. Also the subject matter of claims 20-23 appears to be novel with regard to D2. However, the mutants DN101-104 which are also disclosed in D2 appear to fall under the wording of claims 18 and 19 which therefore lack novelty (Art. 33 (2) PCT).

4). Inventive Step

The problem underlying the present application is that bacterial cultures, used for example for the fermentation of milk, are often subject to bacteriophage infection. This problem is solved by the provision of bacteria that are purine or pyrimidine mutants but still capable of metabolizing substrate material.
It is common knowledge that DNA replication, RNA transcription and protein synthesis is necessary for bacteriophage development.
However, with regard to the description page 17, lines 11-13, it was apparently not known that purine auxotrophic mutants of L.lactis not growing in milk would be capable of acidifying such a substrate material.
Therefore the use of a purine auxotrophic mutant of L.lactis in the method of claims 1-4, 6-11and 27 involves an inventive step (overcoming of a prejudice).

The use of a pyrimidine (Pyr$^-$) mutant is not obviously derivable from the prior art. Therefore claims 5 and 20-27 are inventive.

For claims 12-17 it has not been shown that the genetically modified strain indeed solves the problem of not being susceptible to attack by bacteriophages due to not being capable of DNA replication, RNA transcription or protein synthesis and that such strain has enhanced metabolic activity relative to its parent strain. Since these features do not seem to be sufficiently disclosed in the application, an inventive step cannot be acknowledged for the subject matter of claims 12-17.

5). The applicant is requested to file amendments by way of replacement pages in the manner stipulated by Rule 66.8(a) PCT. In particular, fair copies of the amendments should be filed preferably in triplicate.

Moreover, the applicant's attention is drawn to the fact that, as a consequence of

Rule 66.8(a) PCT the examiner is not permitted to carry out any amendments under the PCT procedure, however minor these may be.

Any information the applicant may wish to submit concerning the subject-matter of the invention, for example further details of its advantages or of the problem it solves, and for which there is no basis in the application as filed, should be confined to the letter of reply and not be incorporated into the application (Article 34(2)(b) PCT).

In order to facilitate the examination of the conformity of the amended application with the requirements of Article 34(2)(b) PCT, the applicant is requested to clearly identify the amendments carried out, no matter whether they concern amendments by addition, replacement or deletion, and to indicate the passages of the application as filed on which these amendments are based (see also Rule 66.8(a) PCT).

If the applicant regards it as appropriate these indications could be submitted in handwritten form on a copy of the relevant parts of the application as filed.

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Correspondence with the EPO on PCT Chapter II demands

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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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Published

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(54) Title: METHOD OF PREVENTING BACTERIOPHAGE INFECTION OF BACTERIAL CULTURES

(57) Abstract

Method of preventing bacteriophage infection of bacterial cultures comprising modified strains, wherein the cultures are completely resistant to bacteriophage attack and have retained their capability of being metabolically active. The method is useful in the manufacturing of food products, feed products or useful metabolite products.

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METHOD OF PREVENTING BACTERIOPHAGE INFECTION OF BACTERIAL CULTURES

FIELD OF INVENTION

The present invention relates to the field of bacterial cultures which are used industrially in the manufacturing of e.g. food products or useful metabolite products. In particular there is provided modified bacterial strains, which, when cultivated in a selected substrate material, are not susceptible to attack by bacteriophages, and have retained their capability of being metabolically active.

TECHNICAL BACKGROUND AND PRIOR ART

Production failures of bacterial cultures caused by bacteriophage infection are considered to be one of the major problems in industrial use of bacterial cultures. Bacteriophages have been found for many of the bacterial strains used in the industry, such as species of lactic acid bacteria e.g. *Lactococcus* spp., *Lactobacillus* spp., *Leuconostoc* spp., *Pediococcus* spp., and *Streptococcus* spp., *Propionibacterium* spp., *Bifidobacterium* spp, *Staphylococcus* spp. or *Micrococcus* spp. Furthermore, bacteriophage infections are also well known in other industrially useful species such as *Bacillus* spp., *Enterobacteriaceae* spp. including *E. coli*, *Corynebacterium* spp. *Actinomycetes* spp. and *Brevibacterium* spp.

In the food industry lactic acid bacterial starter cultures are widely used for food fermentations. It appears that among members of the lactic acid bacteria *Lactococcus* spp. are most devastated by bacteriophage infections. A factor, which leads to frequent bacteriophage infections in lactic acid bacterial starter cultures is the fact that the fermentation conditions in the food industry including the dairy industry are generally non-sterile. Thus, it has not yet been possible to eliminate bacteriophage contamination under these industrial conditions.

The lytic development of phages involves adsorption of the phages to the host cell surface, injection of phage DNA into the cell, synthesis of phage proteins, replication

of phage DNA, assembly of progeny phages and release of progeny from the host. Cell-mediated mechanisms of interference with any of these events can prevent a phage infection. The ability of bacterial cultures to resist bacteriophage infection during industrial use depends to a large extent on host strain characteristics affecting one or more of the above mechanisms.

Thus, it has been shown that natural bacteriophage resistance or defense mechanisms exist in bacterial strains which ensure a certain level of protection against bacteriophage attack. These natural defense mechanisms include phage adsorption inhibition, prevention of phage DNA penetration, restriction of phage DNA and abortive infection.

The prevention of productive contact between phages and bacterial cells due to altered cell surface receptors for phages greatly reduces the ability of the phages to attack the cells. Adsorption of the phages to the cell surface is not always sufficient for the translocation of the phage DNA. It has been shown that host specific cell membrane proteins are involved in the prevention of phage DNA penetration.

Restriction/modification is a mechanism that operates by the cooperation of two enzyme systems, a DNA-cleaving restriction enzyme and a DNA-modifying enzyme, usually a methylase. The mechanism functions by cleaving the phage DNA, as it enters the cell.

Abortive phage infection is described as a mechanism that interferes with the phage development after phage expression has begun. This may eventually lead to a reduced level or termination of the production of viable phage progeny.

However, like many other traits of bacterial strains which are important for industrial performance, the above described natural phage defense mechanisms have been shown to be unstable characteristics, as they may be mediated by plasmids. Furthermore, these defense mechanisms are often phage specific, i.e. they are only active against a limited range of bacteriophage types. Accordingly, the prior art is not aware of a general and stably maintained host cell associated resistance mechanism against bacteriophage infection.

Based on the above natural defense mechanisms, the industry has designed and implemented strategies to possibly reduce bacteriophage infection of bacterial cultures including starter cultures for the fermentation of dairy products. Currently used strategies include the use of mixed starter cultures and alternate use of strains having different phage susceptibility profiles (strain rotation).

Traditionally, starter cultures in the dairy industry are mixtures of lactic acid bacterial strains. The complex composition of mixed starter cultures ensures that a certain level of resistance to phage attack is present. However, repeated subculturing of mixed strain cultures leads to unpredictable changes in the distribution of individual strains and eventually undesired strain dominance. This in turn may lead to increased susceptibility to phage attack and risk of fermentation failures.

Rotation of selected bacterial strains which are sensitive to different phages is another approach to limit phage development. However, it is difficult and cumbersome to identify and select a sufficient number of strains having different phage type profiles to provide an efficient and reliable rotation program. In addition, the continuous use of strains requires careful monitoring for new infectious phages and the need to quickly substitute a strain which is infected by the new bacteriophage by a phage resistant strain. In manufacturing plants where large quantities of bulk starter cultures are made ahead of time, such a quick response is usually not possible.

Studies have shown that a reduced growth capacity of a bacterial culture such as a proteinase-deficient lactic acid bacterium results in reduced phage proliferation (Richardson et al., 1983, 1984). However, such bacterial strains are still growing and are thus still susceptible to attack by phages.

Thus, the industry is not in the possession of any reliable strategy to secure that bacterial cultures used for industrial manufacturing of food products or other products are resistant against attack by bacteriophages. Furthermore, none of the currently used strategies prevent infections by any type of bacteriophages and none of these strategies are capable of precluding that bacteriophages, by a mutational event, circumvent the resistance mechanisms of the bacterial culture strains.

It is therefore a significant objective of the present invention to provide a method of preventing bacteriophage infection of bacterial cultures which are used in the manufacturing of food products and other products, wherein the cultures are completely resistant to attack by all types of bacteriophages.

SUMMARY OF THE INVENTION

Accordingly, the invention provides in a first aspect a method of modifying a substrate material by means of a bacterial culture which is capable of being metabolically active in said substrate, whereby the bacterial culture is not susceptible to attack by bacteriophages, the method comprising

(i) isolating a bacterial strain which is not capable of DNA replication, RNA transcription or protein synthesis in said substrate material but is capable of metabolically modifying the substrate material,

(ii) propagating the selected strain in a medium wherein the strain is capable of replicating to obtain a culture of said strain,

(iii) adding the thus obtained bacterial culture to the substrate material and keeping the material under conditions where the culture is metabolically active,

whereby, if the substrate material is contaminated with a bacteriophage, the metabolic activity of the bacterial culture is substantially unaffected by the bacteriophage.

The invention pertains in another aspect to a modified lactic acid bacterium that is modified to become incapable of performing DNA replication, RNA transcription or protein synthesis in a specifically defined substrate material which is limited with respect to at least one compound that is required by the bacterial strain for DNA replication, RNA transcription or protein synthesis, said modified bacterial strain is capable of being metabolically active in said substrate material, whereby the strain is

not susceptible to attack by bacteriophages, subject to the limitation, that the lactic acid bacterium is not strain DN105 (DSM12289).

In further aspects, there is provided a starter culture compostion comprising the lactic acid bacterium according to the invention and a starter culture compostion comprising a lactic acid bacterium obtainable by the method according to the invention in combination with at least one further lactic acid bacterium.

In a further aspect, there is provided a method of manufacturing a food or feed product comprising adding a starter culture composition according to the invention to a food or feed product starting material and keeping the thus inoculated starting material under conditions where the lactic acid bacterium is metabolically active.

The invention relates in a further aspect to a method of preventing that a lactic acid bacterial starter culture is infected by bacteriophages in the manufacturing of a food or feed product, the method comprising adding as a starter culture a lactic acid bacterium obtained by the method according to the invention to a food or feed product starting material which is limited with respect to at least one compound that is required by the bacterial strain for DNA replication, RNA transcription or protein synthesis and keeping the thus inoculated starting material under conditions where the lactic acid bacterium is metabolically active, whereby, if the substrate material is contaminated with a bacteriophage, the metabolic activity of the bacterial culture is substantially unaffected by the bacteriophage.

In a still further aspect, the invention pertains to the use of a culture as obtained in the method of the invention or a lactic acid bacterium according to the invention as a starter culture in the preparation of a product selected from the group consisting of a dairy flavour, a product for cheese flavouring, a food product and a feed product.

DETAILED DISCLOSURE OF THE INVENTION

Thus, in its broadest aspect the invention provides a method of modifying a substrate material by means of a bacterial culture which is capable of being metabolically active

in said substrate, whereby the bacterial culture is not susceptible to attack by bacteriophages, said method comprising the steps (i) to (iii) as mentioned above. As used herein, the expression "modifying a substrate material" is used interchangeably with the term "fermentation" and relates to any aerobic or anaerobic breakdown of organic compounds by a bacterial culture with the production of an end product. In addition, it will be appreciated that the expression "metabolically active" refers to the capability of the bacterial culture to convert a substrate material such as e.g. milk or a sugar.

In the present context, the expression "not susceptible to attack by bacteriophages" includes the capability of a host cell to be metabolically active even though a bacteriophage adsorbs to the host cell surface and injects its DNA into the host cell. As used herein, the term "bacteriophages" referes to any kind of virus that infects bacteria, including the group of prolate headed bacteriophages, isometric headed bacteriophages and group P335 of bacteriophages.

In accordance with the invention, the method comprises in one aspect the isolation of a bacterial strain which is not capable of DNA replication, RNA transcription or protein synthesis in a specifically defined substrate material, but is capable of metabolically modifying said material. It will be understood that in this context the expression "a specifically defined substrate material" referes to substrate material which is limited with respect to at least one nutrient compound that is required by the bacterial strain for DNA replication, RNA transcription or protein synthesis. Evidently, it was a very surprising finding that it is possible to provide such non-proliferating bacterial strains which are unable to grow in specifically defined substrate materials, but which have retained their capability of being metabolically active. As used herein, the expression "non-proliferating bacterial strain" relates to a bacterial strain which is incapable of multiplying in a specifically defined substrate material.

In a particularly useful embodiment of the present invention the above specific substrate material is limited with respect to at least one nutrient compound that is required by the bacterial strain for DNA replication, RNA transcription or protein synthesis. Such compounds include amino acids or nitrogenous bases such as purine and pyrimidine bases.

Thus, the growth of the bacterial strain is prevented due to the lack of capability of the strains to synthesize the specific compound with respect to which the substrate is limited. Such a mutant strain which has lost the capability of *de novo* synthesising such essentially compounds is also referred to in the art as an "auxotrophic strain". Therefore, in preferred embodiments, the bacterial strain is a mutant strain being auxothrophic in respect of a compound which is not present in the substrate material and which is required by the strain for DNA replication, RNA transcription or protein synthesis.

The substrate material used in the method of the invention may in a further useful embodiment contain at least one compound that inhibits the DNA replication, RNA transcription or the protein synthesis of the bacterial strain. Examples of such compounds include chloramphenicol and erythromycin which affect the ribosomes of the bacterial cell and thus inhibit protein synthesis.

In accordance with the invention, the propagation of the selected strain prior to its use in the present method requires a medium wherein the strain is capable of replicating to obtain a culture of said strain. It is assumed that a medium containing the specific compound which the mutant is unable to synthesize, will restore the capability of the mutant to grow, i.e. capability of DNA replication, RNA transcription and/or protein synthesis.

In a further step of the method according to the invention, the above obtained bacterial culture is added to the above substrate material and kept under conditions where the culture is metabolically active. It will be understood that in this context, the term "conditions" includes the temperature, pH, appropriate composition of the substrate material or presence/absence of an inducer substance, at which the metabolic activity of the bacterial culture is optimal.

Bacteriophages require hosts with intact DNA replication, RNA transcription and protein synthesis in order to become proliferated. Accordingly, bacterial cultures used in the method of the invention are incapable of performing one of the above activities, which makes such bacterial cultures substantially completely resistant to attack by

bacteriophages. Thus, the metabolic activity of the bacterial culture is substantially unaffected even if the substrate material is contaminated with bacteriophages. As used herein, the expression "substantially unaffected" indicates that by using conventional detection methods no changes or only minor changes in the metabolic activity can be detected.

It will be appreciated that such auxotrophic bacteria can be provided by subjecting a wild type bacterial strain that, under appropriate conditions, is capable of growing in a substrate material with or without a specific compound needed for DNA replication, RNA transcription or protein synthesis to a mutagenization treatment and selecting a strain that is substantially incapable of growing in the absence of said specific compound.

Suitable mutagens include conventional chemical mutagens and UV light. Thus, as examples, a chemical mutagen can be selected from (i) a mutagen that associates with or becomes incorporated into DNA such as a base analogue, e.g. 2-aminopurine or an interchelating agent such as ICR-191, (ii) a mutagen that reacts with the DNA including alkylating agents such as nitrosoguanidine or hydroxylamine, or ethane methyl sulphonate (EMS).

As an alternative, auxotrophic bacteria can be provided by selecting spontaneously occurring mutants which, compared to the parent strain, has a growth requirement for a compound needed for DNA replication, RNA transcription or protein synthesis.

It will be understood that it is also possible to provide an auxotrophic mutant by site-directed mutagenesis, e.g. by using recombinant DNA techniques, such as gene knock-out techniques, by which the specific gene is disrupted and rendered non-functional in the bacterium. It is also possible to construct the mutated bacterial strains according to the method of the present invention by techniques which involve the loss of part of the chromosome or a nucleotide base or bases in the DNA sequence which renders the specific gene non-functional in the bacterium. An illustrative example of such a deletion strategy is described in details in the below Examples.

The above bacterial wild type parent strain can be selected from any industrially suitable bacterial species, i.e. the strain can be selected from the group consisting of *Lactococcus* spp. including *L. lactis*, *Lactobacillus* spp., *Leuconostoc* spp., *Pediococcus* spp., *Streptococcus* spp., *Propionibacterium* spp., *Bifidobacterium* spp., *Staphylococcus* spp., *Micrococcus* spp., *Bacillus* spp., *Actinomycetes* spp., *Enterobacteriaceae* spp. including *E. coli*, *Corynebacterium* spp. and *Brevibacterium* spp.

In one specific embodiment of the method according to the invention, a Pur⁻ mutant, including the *Lactococcus lactis* strain DN105 (DSM 12289), is used. This acidifying bacterial strain DN105 is a purine auxotrophic mutant capable of acidifying milk, even though the strain is not capable of growth because of its requirement for purine, which is not present in milk in sufficient amounts to support growth of such a bacterium. As it is shown in the below Example 1, strain DN105 is capable of acidifying milk under purine starvation conditions even in the presence of a high concentration of bacteriophages.

In a further useful embodiment of the method according to the invention, a *thyA* mutant, including the *Lactococcus lactis* strain MBP71 (DSM12891), is used. This acidifying bacterial strain MBP71 is a thymidine auxotrophic mutant, which is capable of acidifying milk, even though the strain is not capable of growth, due to its requirement for thymidine, which is not present in milk in sufficient amounts to support growth of such a mutant. As shown in Example 2 below, strain MBP71 is capable of acidifying milk under thymidine starvation conditions even in the presence of a high concentration of bacteriophages.

It is convenient to provide the above bacterial strains both when used as a food or feed production strain and as a production strain for metabolites, as a composition comprising the bacterial strain selected for the specific use. Typically, such compositions contain the bacterium in concentrated form e.g. at a concentration of viable cells (colony forming units, CFUs) which is in the range of 10^5 to 10^{13} per g of the composition such as in the range of 10^6 to 10^{12} per g. Additionally, the culture composition may contain further components such as bacterial nutrients, cryoprotectants or other substances enhancing the viability of the bacterial active ingredient

during storage. The composition can e.g. be in the form of a liquid, frozen or a freeze-dried composition.

As mentioned above, one characteristic of the bacterial culture as used in the method of the invention, is that it is capable of metabolically modifying a specifically defined substrate material even though the strain is incapable of growth in such substrate. When a lactic acid bacterium is used in the method of the invention, the obtained bacterial culture typically has an acidification rate in milk which is at least 10% of that of said culture when it is present in a substrate material where it is capable of DNA replication, RNA transcription and/or protein synthesis. In preferred embodiments, the bacterial strain has an acidification rate in milk which is at least 1% including at least 5%, such as at least 10%, e.g. at least 15%, such as at least 20%, including at least 25% of that of the culture when it is present in a substrate material where it is capable of DNA replication, RNA transcription and/or protein synthesis.

Typically, the bacterial strain used in the method of the invention is added to the substrate material at a concentration in the range of 10^5 to 10^9 CFU/ml or g of the material, such as at least 10^5 CFU/ml or g of the material, including at least 10^6 CFU/ml or g of the material, such as at least 10^7 CFU/ml or g of the material, e.g. at least 10^8 CFU/ml or g of the material, including at least 10^9 CFU/ml or g of the material.

Thus, the obtainment of a bacterial culture which is completely resistant to bacteriophage attack according to the method of the invention, can be utilized in all industrial contexts where proliferation of the culture in the substrate material is not a requirement. Dairy fermentations of milk is such an example of an industrial manufacturing process, as proliferation of the lactic acid bacteria during milk fermentation is not a requirement, if the desired taste and acidification of the fermentation product are obtained. Therefore, in useful embodiments, the substrate material is a starting material for an edible product including milk, a vegetable material, a meat product, a must, a fruit juice, a wine, a dough and a batter.

In further embodiments, the substrate material is a starting material for an animal feed such as silage e.g. grass, cereal material, peas, alfalfa or sugar-beet leaf, where

bacterial cultures are inoculated in the feed crop to be ensiled in order to obtain a preservation hereof, or in protein rich animal waste products such as slaughtering offal and fish offal, also with the aims of preserving this offal for animal feeding purposes.

Yet another significant application of the method according to the present invention is the use of the bacterial cultures as so-called probiotics. By the term "probiotic" is in the present context understood a microbial culture which, when ingested in the form of viable cells by humans or animals, confers an improved health condition, e.g. by suppressing harmful microorganisms in the gastrointestinal tract, by enhancing the immune system or by contributing to the digestion of nutrients.

It is, as mentioned above, an important objective of the present invention to provide a method of preventing bacteriophage infection of bacterial cultures which are metabolically active by using non-proliferating bacterial cells. In order to be of industrial interest, such metabolic activity should result in the production of a substantial amount of the desired end product. Thus, one possibility of increasing such production by use of a non-proliferating cell is an enhancement of the flux through metabolic pathways.

Accordingly, in one useful embodiment, the bacterial culture used in the method of the invention comprises a genetically modified strain which, relative to its parent strain, is enhanced in at least one metabolic pathway. Such enhanced metabolic activity can e.g. be obtained through an enhanced glycolytic pathway and/or an enhanced flux through the pentose phosphate pathway.

One approach for stimulating the flux through the glycolytic pathway is by increasing the expression of ATPase activity, i.e. an enhanced conversion of ATP to ADP, as described in WO 98/10089. Thus, in one useful embodiment of the invention, the genetically modified strain has, relative to its parent strain, an enhanced ATPase activity.

In one interesting embodiment of the present invention, the genetically modified strain is one wherein the gene coding for an ATPase is under the control of a regulatable promoter. As used herein, the term "regulatable promoter" is used to describe a

promoter sequence possibly including regulatory sequences for the promoter, which promoter is regulatable by one or more factors present in the environment of the strain. Such factors include the pH of the growth medium, the growth temperature, a temperature shift eliciting the expression of heat shock genes, the composition of the growth medium including the ionic strength/NaCl content and the growth phase/growth rate of the bacterium. Such a regulatable promoter may be the native promoter or it may be an inserted promoter not naturally related to the gene either isolated from the same bacterial species or it may be a heterologous promoter sequence, i.e. a sequence derived from different bacterial species.

Cells such as "resting cells" or "non-dividing cells" represent other types of non-proliferating cells which are useful in the method of the invention and wherein the above enhancement of the flux through the metabolic pathways is useful. Such cells are incapable of mitosis or meiosis e.g. due to the deficiency of DNA, RNA and/or protein needed for the separation of the cell. Thus, in a particularly useful embodiment, the bacterial culture is one which comprises a bacterial strain which is capable of increasing the size of the cells without mitosis.

In addition, the invention encompasses non-proliferating strains which under specific conditions are incapable of growth. Thus, in an interesting embodiment, the bacterial culture comprises a strain which is a conditional mutant, i.e. a mutant which under predetermined conditions does not perform at least one activity selected from the group consisting of DNA replication, RNA transcription and protein synthesis. Such predetermined conditions include pH, temperature, composition of the substrate material and presence/absence of inducer substances.

One possible means of providing such conditional mutants which are temperature-sensitive is by subjecting a bacterial strain that under appropriate conditions is capable of growing in a substrate material e.g. at a temperature below 30°C to a mutagenization treatment and selecting a mutant strain that is substantially incapable of growth at temperatures below 30°C, but is capable of growth at higher temperatures.

In a further aspect of the invention there is provided a modified lactic acid bacterium that is modified to become incapable of performing DNA replication, RNA transcription or protein synthesis in a specifically defined substrate material which is limited with respect to at least one compound that is required by the bacterial strain for DNA replication, RNA transcription or protein synthesis, said modified bacterial strain is capable of being metabolically active in said substrate material, whereby the strain is not susceptible to attack by bacteriophages, subject to the limitation, that the lactic acid bacterium is not strain DN105 (DSM12289).

In useful embodiments, the lactic acid bacterium according to the invention is a mutant strain being auxothrophic in respect of a compound which is not present in the substrate material and which is required by the strain for replication.

Presently preferred lactic acid bacteria according to the invention are mutant strains which are *thyA* mutants including *Lactococcus lactis* strain MBP71 deposited under the accession number DSM12891.

The modified lactic acid bacterium according to the invention is useful as a starter culture in the production of food and feed products. Accordingly, in a further important aspect the invention relates to a starter culture composition comprising the lactic acid bacterium according to the invention.

As it is normal in the production of lactic acid bacterial fermentation processes to apply mixed cultures lactic acid bacteria, a composition will in certain embodiments comprise a multiplicity of strains either belonging to the same species or belonging to different species. Accordingly, in a further important aspect, the invention relates to a starter culture composition comprising a lactic acid bacterium obtainable by the method according to the invention in combination with at least one further lactic acid bacterium. A typical example of such a useful combination of lactic acid bacteria in a starter culture composition is a mixture of the bacterium obtainable by the method according to the invention and one or more *Lactococcus* spp. such as *Lactococcus lactis* subsp. *lactis* or *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* or *Leuconostoc* spp. Such a mixed culture can be used in the manufacturing of fermented milk products such as buttermilk and cheese.

In one embodiment, the composition according to the invention, is one which further comprising at least on component enhancing the viability of the bacterial active ingredient during storage including a bacterial nutrient or a cryoprotectant.

It is also an objective of the present invention to provide a method of manufacturing a food or feed product based on the use of the modified bacterium of the invention. Thus, in its broadest aspect, such a method comprises adding a starter culture composition according to the invention to a food or feed product starting material and keeping the thus inoculated starting material under conditions where the lactic acid bacterium is metabolically active. In a particularly useful embodiment, the food product starting material is milk.

In further aspects, the invention relates to the use of a culture as obtained in the method according to the invention as a starter culture in the preparation of a product selected from the group consisting of a dairy flavour, a product for cheese flavouring, a food product and a feed product.

The invention will now be described in further details in the following non-limiting examples and the drawings wherein

Fig. 1. shows the acidification of reconstituted skim milk (RSM) by strain DN105. The pH was followed over time in RSM cultures containing strain *Lactococcus lactis* DN105 inoculated at 1%, 10%, 25% and 50% vol/vol;

Fig. 2 illustrates the development of pH in RSM when inoculated with 25% vol/vol of *Lactococcus lactis* strain CHCC373 or *Lactococcus lactis* strain DN105, in the presence of the bacteriophage strain 836 at a concentration of at least 10^8 PFU/ml;

Fig. 3A-3H illustrate the development of pH in RSM when inoculated with 25% vol/vol of *Lactococcus lactis* strain DN105 with or without addition of the purine compound hypoxanthine to the culture medium, in the presence of at least 10^8 PFU/ml of the following bacteriophages: CHPC836 (3A); CHPC412 (3B); CHPC783 (3C); CHPC795 (3D); CHPC710 (3E); CHPC12 (3F); CHPC708 (3G); CHPC814 (3H);

Fig. 4. shows the acidification of RSM by *Lactococcus lactis* strain MBP71 inoculated at different concentrations. The pH of 200 ml RSM cultures comprising strain MBP71 inoculated at 1%, 5%, 10%, 25%, and 50% (v/v) was monitored on-line; and

Fig. 5. shows the acidification of RSM, comprising bacteriophages and/or thymidine, by *Lactococcus lactis* strains MBP71 and CHCC373, which were inoculated at a concentration of 25% (v/v) in a volume of 100 ml RSM. The thymidine concentration was 20 mg/l and bacteriophage CHPC733 was added to a concentration of $2x10^8$ PFU/ml. The pH was monitored by collecting 2.5 ml samples.

EXAMPLE 1

The use of a purine auxotrophic *Lactococcus lactis* strain for obtaining resistance against bacteriophages in milk fermentations

1.1. Materials and methods

(i) Bacterial strains, media and growth conditions

The *Lactococcus lactis* strain DN105 is a purine auxotrophic mutant (Pur$^-$) derived from the wild type strain CHCC373 described in Nilsson and Lauridsen (1992). A sample of *Lactococcus lactis* strain DN105 is deposited with Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany on 30 June 1998 under the accession No. DSM 12289.

Lactococcus lactis was grown in M17 (Terzaghi & Sandine, 1975) in the chemically defined DN medium (Dickely et al., 1995) or in reconstituted skim milk (RSM) containing 9.5% (w/vol) low fat skim milk powder at 30°C. For the propagation of strain DN105, the medium was supplemented with hypoxanthine to a final concentration of 15-50 mg/l. The Pur$^-$ phenotype of strain DN105 was tested by its ability to grow on DN medium with and without hypoxanthine supplement.

(ii) Bacteriophages and their handling

Bacteriophages were purified by three single plaque isolation steps. The high titer bacteriophage lysates were prepared by consecutive infections of the host strain CHCC373 with the bacteriophage at an MOI (multiplicity of infection) of 0.1 to 1.0. After infection the culture was grown at 30°C in M17 supplemented with 10 mM $CaCl_2$ until completed lysis. The lysates were centrifuged for 15 min at 6.000 rpm and the supernatant sterile filtered (0.45 μm, Schleicher und Schuell).

(iii) Determination of bacteriophage titers

For the determination of bacteriophage titers (plaque forming units per ml) the agar double layer method was used (Adams M.H., 1959; Interscience Publishers, Inc., New York). The bacteriophages used for the acidification test are listed in Table 1 below:

Table 1. Bacteriophages and phage titers

Phage	Titer (PFU/ml)
CHPC12	$4x10^{10}$
CHPC412	$3x10^{10}$
CHPC708	$2x10^{10}$
CHPC710	$2x10^{10}$
CHPC783	$1x10^{11}$
CHPC795	$7x10^{10}$
CHPC814	$1x10^{10}$
CHPC836	$4x10^{10}$

(iv) Test for the acidification of RSM by strain DN105

10-400 ml of an outgrown culture of strain DN105 in M17 was harvested by centrifugation, washed twice with a sterile solution of 0.9% NaCl to remove residual purine compounds and resuspended in 10-400 ml of RSM to give the same cell

density as in the outgrown M17 culture. The resuspended material was used for inoculation of fresh RSM at volume/volume concentrations typically in the range of 10 to 100% (v/v). The pH was monitored either on-line or by measuring the pH of 3 ml samples collected at intervals.

1.2. Results of the acidification of RSM by *Lactococcus lactis* strain DN105

In general, lactic acid bacterial cells which do not have intact DNA replication, RNA transcription and protein synthesis systems are unable to grow and acidify milk. Nilsson and Lauridsen (1992) demonstrated that the purine auxotrophic mutant DN105 is unable to grow in a medium without purines. It has also been reported that purine auxotrophic mutants of *Lactococcus lactis* not growing in milk are incapable of acidifying such a substrate material (Dickely et al., 1995). To test the ability of strain DN105 to acidify a milk based medium, the strain was inoculated in varying amounts in RSM as described in Materials and Methods and the pH of the substrate material was monitored (Fig. 1).

The results shown in Fig. 1 clearly demonstrates that strain DN105 was able to acidify milk at least to pH 5.0 even under purine starving conditions.

1.3 Studies of the bacteriophage resistance of *Lactococcus lactis* strain DN105 in RSM

As it is generally known, a bacteriophage requires a susceptible host cell which has intact DNA replication, RNA transcription and protein synthesis systems for development. If a potential host is not capable of performing at least one of these activities, bacteriophages cannot be proliferated in the cell.

Fig. 2 shows the development of pH in RSM when inoculated with 25% vol/vol of the wild type strain CHCC373 or the mutant strain DN105 in the presence of the bacteriophage CHPC836 at a concentration of at least 10^8 PFU/ml.

In further experiments the resistance of strain DN105 against various bacteriophages was studied in RSM with and without hypoxanthine added. The bacteriophages were added to RSM at 10^8 to 10^9 bacteriophages/ml.

The addition of hypoxanthine was used as a positive control for the bacteriophage infection, as the addition of this purine compound enabled bacteriophage attack (Fig. 3 A-H). In all cultures without hypoxanthine added strain DN105 acidified the milk to around pH 5.0, whereas with the addition of hypoxanthine none of the cultures reached a pH of below 5.4.

1.4 Discussion

This Example shows that the starvation of a Pur⁻ strain of *L. lactis* for purines causes total resistance to a range of bacteriophages and that the Pur⁻ strain of *L. lactis* is capable of effectively acidifying milk in the presence of a large number of bacteriophages for which the corresponding wild type is susceptible.

Thus, the present Example shows that it is possible to develop strains which under appropriate selected conditions, where the strains are incapable of growth, are completely resistant to bacteriophages and that such strains have retained the metabolic ability to acidify milk. Such a system of bacteriophage resistance can be introduced into any bacteria e.g. *Lactococcus* spp., *Lactobacillus* spp., *Leuconostoc* spp., *Pediococcus* spp., *Streptococcus* spp., *Propionibacterium* spp., *Bifidobacterium* spp., *Staphylococcus* spp., *Micrococcus* spp., *Bacillus* spp., *Actinomycetes* spp., *Enterobacteriaceae* spp. including *E. coli*, *Corynebacterium* spp. and *Brevibacterium* spp. in regard to e.g. production of a bacterial fermentation product such as lactate, diacetyl, acetoin, methanethiol, ethanol etc. if proliferation of the bacteria is not a requirement.

EXAMPLE 2

The use of a thymidine auxotrophic *Lactococcus lactis* strain for obtaining resistance against bacteriophages in milk fermentations

2.1. Materials and methods

(i) Bacterial strains, bacteriophages, media and growth conditions

The *Lactococcus lactis* strain MBP71 is a thymidine auxotrophic mutant (*thyA*) derived from the wild type strain CHCC373 (see Example 1). A sample of strain MBP71 has been deposited with Deutsche Sammlung von Mikroorganism und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany on 25 June 1999 under the accession No. DSM12891.

Strain CHCC373 was routinely grown at 30°C in M17 (Terzaghi & Sandine, 1975) supplemented with 0.5% of lactose, or in reconstituted skim milk (RSM) containing 9.5% (w/v) low fat skim milk powder. For the propagation of strain MBP71 the media were supplemented with 20 mg/l of thymidine, since neither M17 nor RSM support the growth of the ThyA$^-$ phenotype. During the manipulations to construct strain MBP71 5 mg/l of erythromycin was added to the M17 based media to maintain the plasmid. Also, when growth at 37°C was required during these manipulations M17 based media were supplemented with 50 mM of NaCl to enhance growth. *E. coli* DH5α (Life Technologies Inc., USA) was grown in LB medium supplemented with 100 mg/l of erythromycin to maintain the transformed plasmid. The bacteriophage CHPC733 was used in growth experiments where strain MBP71 phage resistance was tested. For the bacteriophage handling and determination of titers see Example 1.

(ii) Construction of plasmid with deletion in *thyA* from strain CHCC373

An about 800 bp fragment upstream of *thyA* from strain CHCC373 was obtained by PCR on chromosomal DNA using the primers

TATAATCTGCAGGGTCACACTATCAGTAATTG (SEQ ID NO:1) and
TATTTTAAGCTTCACAGTCTGCTATTTTGATTC (SEQ ID NO:2),

which furthermore introduce a *Pst*I and a *Hin*dIII site, respectively, in the fragment ends. The resulting fragment includes the -35 box of the *thyA* promoter, but not the -

10 box. Another about 800 bp fragment comprising an internal part of the *thyA* coding region was obtained by PCR on chromosomal DNA using the primers

TAAATTAAGCTTCGCAGACAAGATTTTTAAAC (SEQ ID NO:3) and

ATTTAAGTCGACGGCTCATAGTCCACAAGTTC (SEQ ID NO:4),

which introduce a *Hin*dIII and a *Sal*I site, respectively. The resulting fragment include the whole coding region of *thyA* except the first 8 bp and the last 34 bp. The two PCR fragments were purified by using the QIAquick PCR purification kit (QIAGEN GmbH, Germany), cut with the indicated enzymes, and purified again. The pGhost9 vector (Maguin et al., 1996) was cut with the restriction enzymes *Pst*I and *Sal*I and purified. Thereafter, about 150 ng of the vector and about 50 ng of each of the two fragments were ligated overnight at 16°C in a total volume of 20µl. From this mixture 10 µl was used to transform *E. coli* DH5α. Plasmid DNA was isolated from possible clones by growing them overnight in LB medium with 100 mg/l of erythromycin and subsequently using 1.5 ml of these cultures with the QIAprep spin miniprep kit protocol (QIAGEN GmbH, Germany). The purified plasmid DNA was cut with *Pst*I and *Sal*I and one of them, pMBP63, yielded a band of approx. 1600 bp. This construct was further verified by PCR with the primers that were used to produce the two fragments, and also with the two outer primers. Finally, the construct was verified by sequencing both strands over the region of the deletion with the two primers

GACTGTTGCCCCATAGCG (SEQ ID NO:5) and

GCTTCGATTTTAGTATATGG (SEQ ID NO:6).

All primers were from TAG Copenhagen A/S, Copenhagen, Denmark.

(iii) Inactivation of the chromosomal *thyA* gene in strain CHCC373

About 200 ng of pMBP63 was used to transform strain CHCC373. By employing the gene replacement feature (Biswas et al., 1993) of the pGhost9 vector the chromosomal *thyA* gene of strain CHCC373 was thereafter successfully inactivated. The resulting strain, MBP71, was shown to be a thymidine auxotrophic mutant.

(iv) Test for acidification of RSM by strains CHCC373 and MBP71

Overnight cultures of M17 with 0.5% lactose and 20 mg/l of thymidine were washed twice in an isotonic solution of the same volume as the overnight culture to remove residual thymidine. The cells were resuspended in a fresh isotonic solution of a volume which was 1/20 of the overnight culture. These resuspended cells were used to inoculate RSM to a given volume identified by the percentage volume of the overnight culture i.e. an inoculation of 100% (v/v) indicates that 5 ml of the resuspended cells have been used to inoculate 100 ml of RSM. The pH was monitored either on-line or by measuring the pH of 2.5 ml samples.

2.2. Results of the acidification of RSM by *Lactococcus lactis* strain MBP71

To test the acidification of RSM by strain MBP71 this strain was inoculated in varying amounts as described in materials and methods. The pH of the substrate material was then monitored on-line over a 20 hour period. The results shown in Fig. 4 clearly demonstrate that MBP71 is able to acidify milk down to at least pH 4.5 under thymidine starvation conditions. When thymidine was added to the RSM, strain MBP71 acidified the substrate material similarly to strain CHCC373 (details not shown).

2.3. Studies of the bacteriophage resistance of *Lactococcus lactis* MBP71 in RSM

As described above, a bacteriophage requires a susceptible host cell, which has intact DNA replication, RNA transcription and protein synthesis systems, for development. If a potential host is not capable of performing just one of these activities, bacteriophages can not proliferate in the cell. The thymidine auxotrophic strain MBP71 is only capable of DNA replication in the presence of thymidine, or another suitable precursor for dTTP. None of these precursors are present in RSM, and strain MBP71 is therefore not susceptible to bacteriophage attack under these conditions.

Fig. 5 shows the development of pH in 100 ml RSM inoculated with strain MBP71 at a concentration of 25% (v/v). When thymidine was present it was added to a final concentration of 20 mg/l. When the bacteriophage CHPC733 was present it was

added to a concentration of $2x10^8$ PFU/ml half an hour after cell inoculation. This gives a MOI (multiplicity of infection) of 0.2. The results in Fig. 5 clearly demonstrate that strain MBP71 acidifies RSM without thymidine down to at least pH 4.6 even in the presence of bacteriophages. However, when thymidine is added to the RSM strain MBP71 becomes susceptible to bacteriophage attack and the milk is only acidified down to pH 5.5, which is also close to the pH that is reached for the wild type strain CHCC373 in the presence of bacteriophages. If the bacteriophages are omitted, but thymidine is added, strain MBP71 acidifies the RSM similarly to strain CHCC373 (see Fig. 5, details not shown).

2.4. Discussion

This example shows that the starvation of a *thyA* strain of *L. lactis* for thymidine causes total resistance to bacteriophage CHPC733 and that the *thyA* strain of *L. lactis* is capable of effectively acidifying milk under these conditions. This is contrary to the wild type, which is not able to acidify the milk to an acceptable level under the same conditions. Although, strain MBP71 has only been tested for bacteriophage resistance to one bacteriophage it is believed that strain MBP71 is resistant to all, or most, types of bacteriophages.

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Applicant's or agent's file reference number	21134 PC 1	International application No.

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 15, line 24

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet [X]

Name of depositary institution

DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

Address of depositary institution (*including postal code and country*)

Mascheroder Weg 1B
D-38124 Braunschweig
Germany

Date of deposit	Accession Number
30 June 1998	DSM 12289

C. ADDITIONAL INDICATIONS (*leave blank if not applicable*) — This information is continued on an additional sheet [X]

As regards the respective Patent Offices of the respective designated states, the applicants request that a sample of the deposited microorganisms only be made available to an expert nominated by the requester until the date on which the patent is granted or the date on which the application has been refused or withdrawn or is deemed to be withdrawn.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (*if the indications are not for all designated States*)

E. SEPARATE FURNISHING OF INDICATIONS (*leave blank if not applicable*)

The indications listed below will be submitted to the International Bureau later (*specify the general nature of the indications e.g., "Accession Number of Deposit"*)

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INDICATIONS RELATING TO DEPOSITED MICROORGANISMS
(PCT Rule 12bis)

Additional sheet

In addition to the microorganism indicated on page 24 of the description, the following microorganism has been deposited with

DSM-Deutsche Sammlung von Mikroorganismen und Cellkulturen GmbH
Mascheroder Weg 1b, D-38124 Braunschweig, Germany

on the dates and under the accession number as stated below:

Accession number	Date of deposit	Description Page No.	Description Line No.
DSM 12891	25 June 1999	19	7

For the above-identified deposited microorganism, the following additional indications apply:

As regards the respective Patent Offices of the respective designated states, the applicants request that a sample of the deposited microorganism stated above only be made available to an expert nominated by the requester until the date on which the patent is granted or the date on which the application has been refused or withdrawn or is deemed to be withdrawn.

CLAIMS

1. A method of modifying a substrate material by means of a bacterial culture which is capable of being metabolically active in said substrate, whereby the bacterial culture is not susceptible to attack by bacteriophages, the method comprising

(i) isolating a bacterial strain which is not capable of DNA replication, RNA transcription or protein synthesis in said substrate material but is capable of metabolically modifying the substrate material,

(ii) propagating the selected strain in a medium wherein the strain is capable of replicating to obtain a culture of said strain,

(iii) adding the thus obtained bacterial culture to the substrate material and keeping the material under conditions where the culture is metabolically active.

whereby, if the substrate material is contaminated with a bacteriophage, the metabolic activity of the bacterial culture is substantially unaffected by the bacteriophage.

2. A method according to claim 1 wherein the substrate material is limited with respect to at least one compound that is required by the bacterial strain for DNA replication, RNA transcription or protein synthesis.

3. A method according to claim 2 wherein the bacterial strain is a mutant strain being auxothrophic in respect of a compound which is not present in the substrate material and which is required by the strain for replication.

4. A method according to claim 3 wherein the mutant strain is a Pur$^-$ mutant including *Lactococcus lactis* strain DN105 deposited under the accession number DSM 12289.

5. A method according to claim 3 wherein the mutant strain is a *thyA* mutant including *Lactococcus lactis* strain MBP71 deposited under the accession number DSM12891.

6. A method according to any of claims 2 to 5 wherein the strain in said substrate material is not capable of performing at least one activity selected from the group consisting of DNA replication, RNA transcription and protein synthesis.

7. A method according to claim 1 wherein the substrate material contains at least one compound that inhibits the DNA replication, RNA transcription or the protein synthesis of the bacterial strain.

8. A method according to claim 1 wherein the substrate material is a starting material for an edible product, the material is selected from the group consisting of milk, a vegetable material, a meat product, a must, a fruit juice, a wine, a dough and a batter.

9. A method according to claim 1 wherein the bacterial culture is selected from the group consisting of *Lactococcus* spp., *Lactobacillus* spp., *Leuconostoc* spp., *Pediococcus* spp., *Streptococcus* spp., *Propionibacterium* spp., *Bifidobacterium* spp., *Staphylococcus* spp., *Micrococcus* spp., *Bacillus* spp., *Enterobacteriaceae* spp. including *E. coli*, *Actinomycetes* spp., *Corynebacterium* spp. and *Brevibacterium* spp.

10. A method according to claim 9 wherein the bacterial culture is of *Lactococcus lactis*.

11. A method according to claim 1 wherein the bacterial strain is added to the substrate material at a concentration in the range of 10^5 to 10^9 CFU/ml or g of the material.

12. A method according to claim 1 wherein the culture comprises a genetically modified strain which, relative to its parent strain is enhanced in at least one metabolic pathway.

13. A method according to claim 12 wherein the genetically modified strain has, relative to its parent strain, an enhanced metabolic activity selected from the group consisting of enhanced glycolytic flux and enhanced flux through the pentose phosphate pathway.

14. A method according to claim 13 wherein the genetically modified strain has, relative to its parent strain, an enhanced ATPase activity.

15. A method according to claim 1 wherein the bacterial culture comprises a strain which is a conditional mutant which at a predetermined condition does not perform at least one activity selected from the group consisting of DNA replication, RNA transcription and protein synthesis.

16. A method according to claim 15 wherein the predetermined condition is selected from the group consisting of pH, temperature, composition of the substrate material and presence/absence of an inducer substance.

17. A method according to claim 1 wherein the culture comprises a bacterial strain which is capable of increasing the size of the cells without mitosis.

18. A modified lactic acid bacterium that is modified to become incapable of performing DNA replication, RNA transcription or protein synthesis in a specifically defined substrate material which is limited with respect to at least one compound that is required by the bacterial strain for DNA replication, RNA transcription or protein synthesis, said modified bacterial strain is capable of being metabolically active in said substrate material, whereby the strain is not susceptible to attack by bacteriophages, subject to the limitation, that the lactic acid bacterium is not strain DN105 (DSM12289).

19. A lactic acid bacterium according to claim 18 wherein the bacterial strain is a mutant strain being auxothrophic in respect of a compound which is not present in the substrate material and which is required by the strain for replication.

20. A lactic acid bacterium according to claim 19 wherein the mutant strain is a *thyA* mutant including *Lactococcus lactis* strain MBP71 deposited under the accession number DSM12891.

21. A starter culture compostion comprising the lactic acid bacterium of any of claims 18-20.

22. A starter culture composition comprising a lactic acid bacterium obtainable by the method according to claim 1 in combination with at least one further lactic acid bacterium.

23. A composition according to claim 22 which further comprising at least on component enhancing the viability of the bacterial active ingredient during storage including a bacterial nutrient or a cryoprotectant.

24. A method of manufacturing a food or feed product comprising adding a starter culture composition according to any of claims 21-23 to a food or feed product starting material and keeping the thus inoculated starting material under conditions where the lactic acid bacterium is metabolically active.

25. A method according to claim 24 wherein the food product starting material is milk.

26. Use of a culture as obtained in the method of claim 1 or a lactic acid bacterium according to any of claims 18-20 as a starter culture in the preparation of a product selected from the group consisting of a dairy flavour, a product for cheese flavouring, a food product and a feed product.

27. A method of preventing that a lactic acid bacterial starter culture is infected by bacteriophages in the manufacturing of a food or feed product, the method comprising adding as a starter culture a lactic acid bacterium obtained by the method according to claim 1 to a food or feed product starting material which is limited with respect to at least one compound that is required by the bacterial strain for DNA replication, RNA transcription or protein synthesis and keeping the thus inoculated starting material under conditions where the lactic acid bacterium is metabolically active, whereby, if the substrate material is contaminated with a bacteriophage, the metabolic activity of the bacterial culture is substantially unaffected by the bacteriophage.

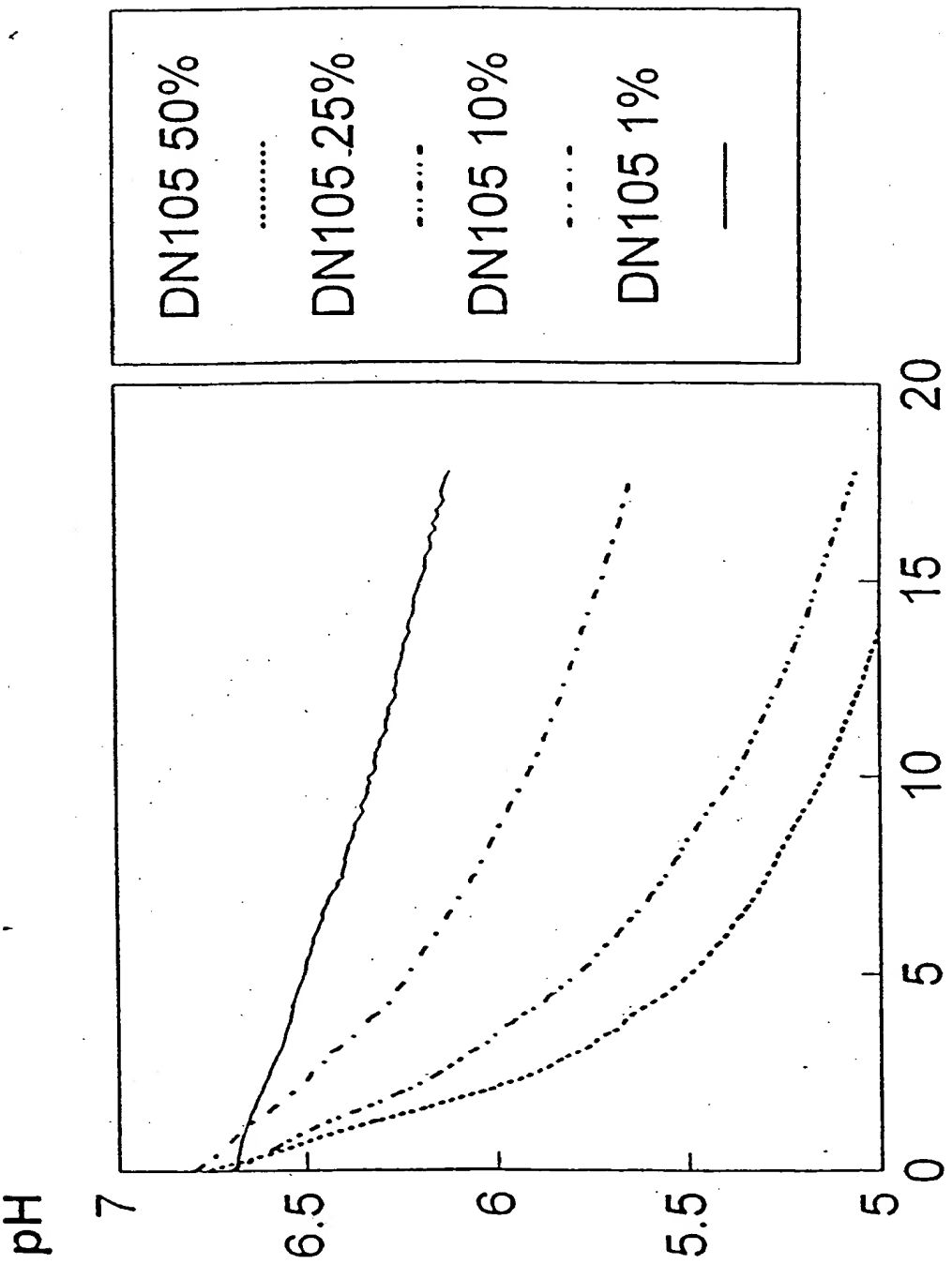


Fig. 1

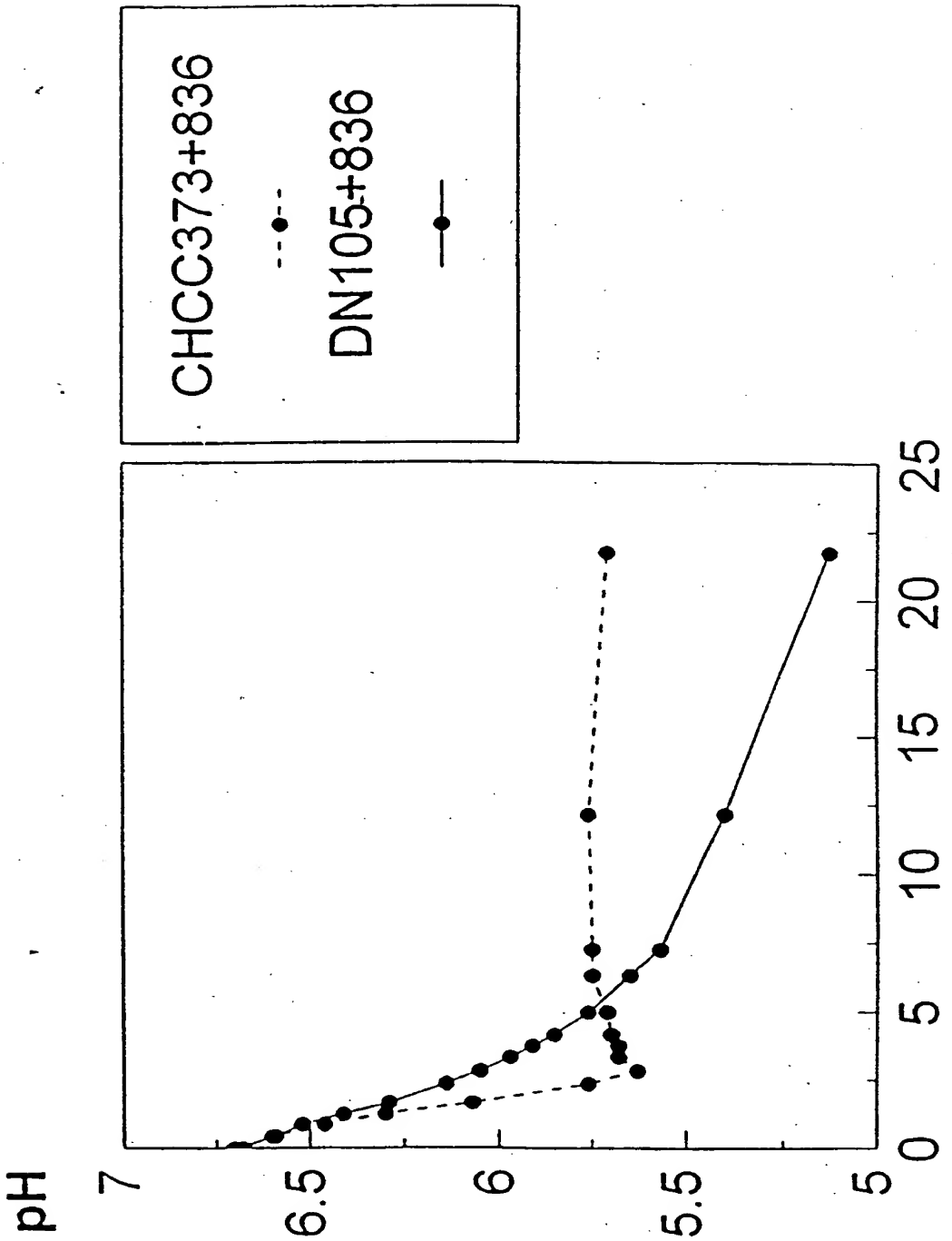
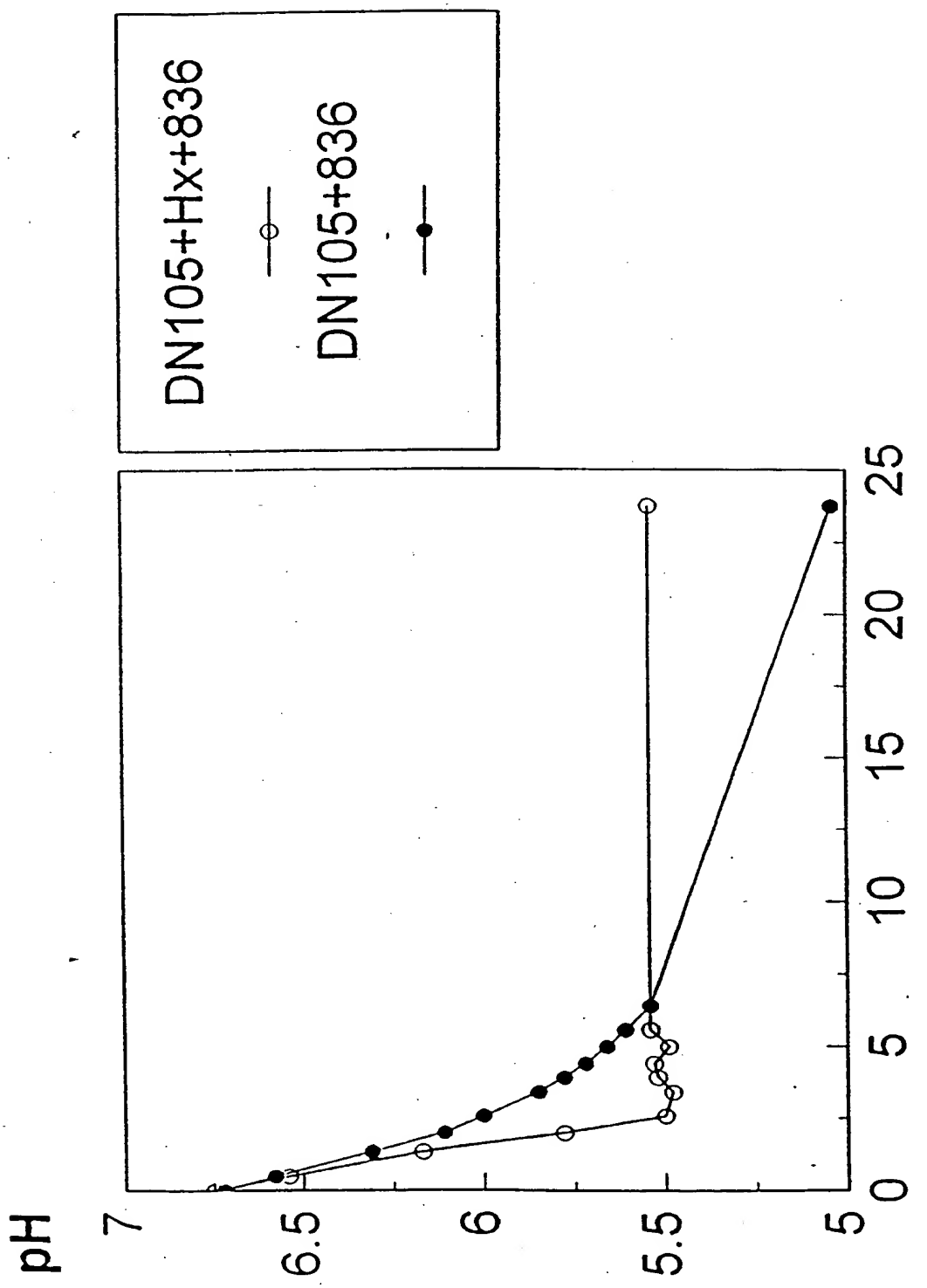


Fig. 2



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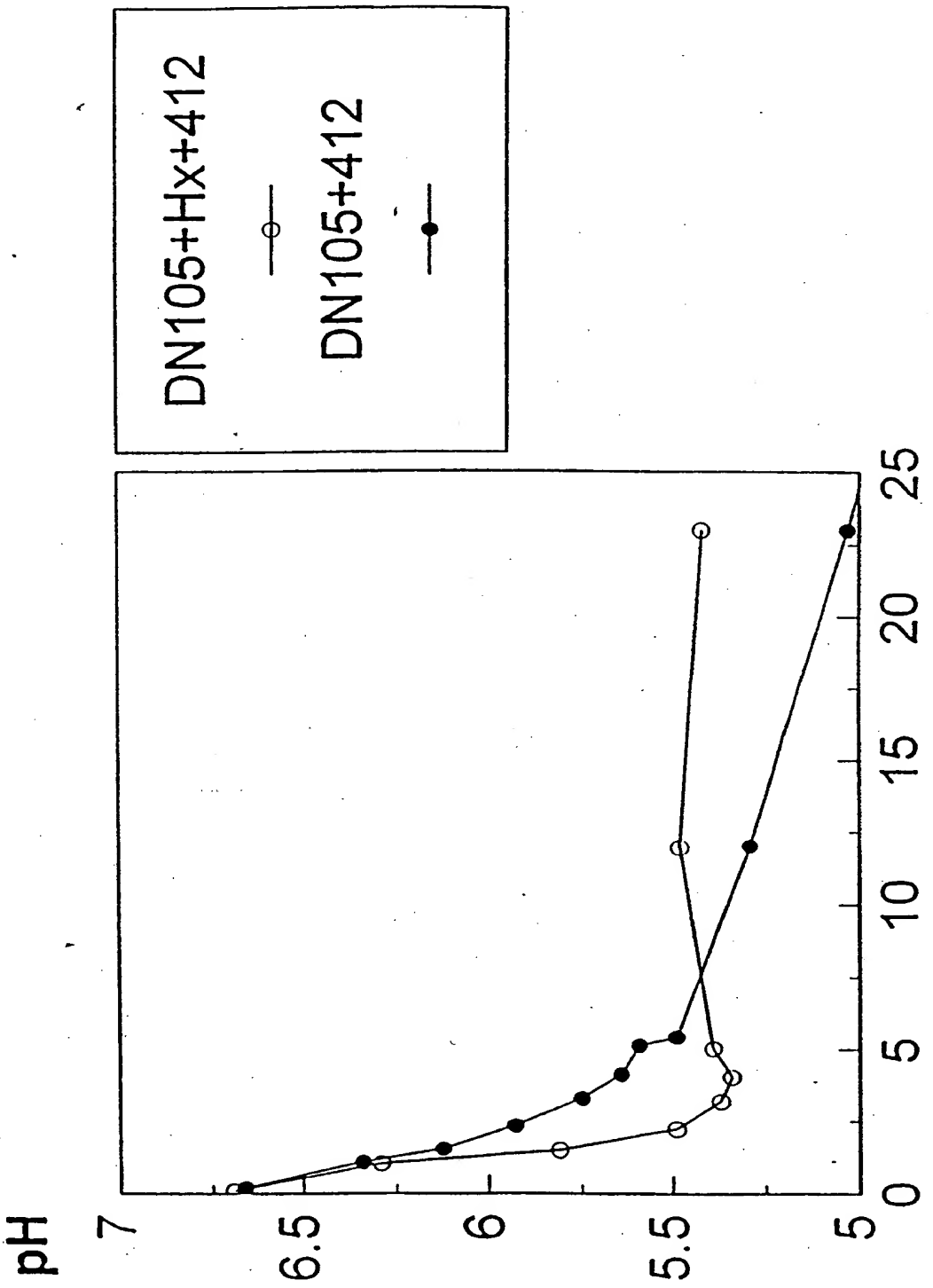

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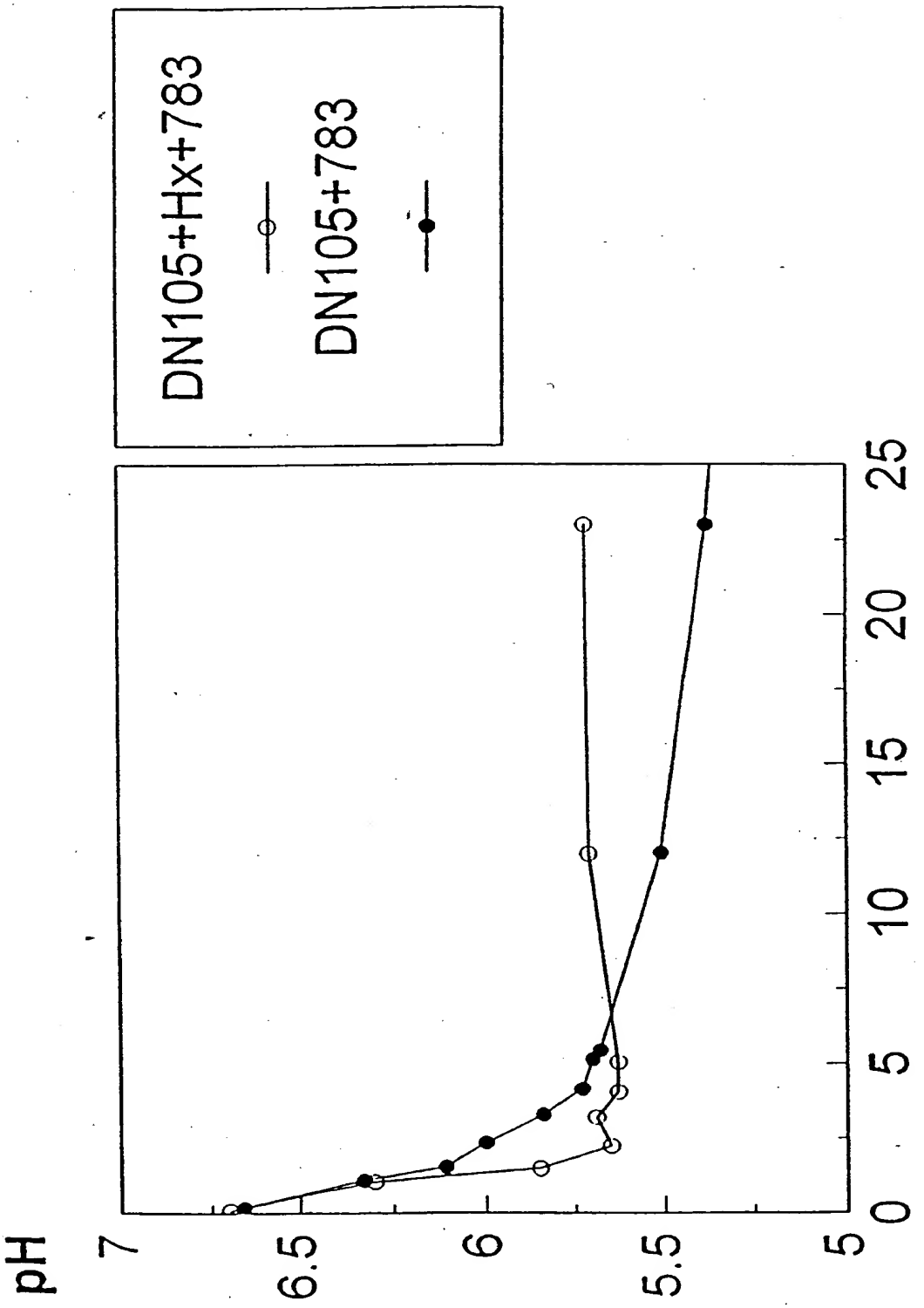

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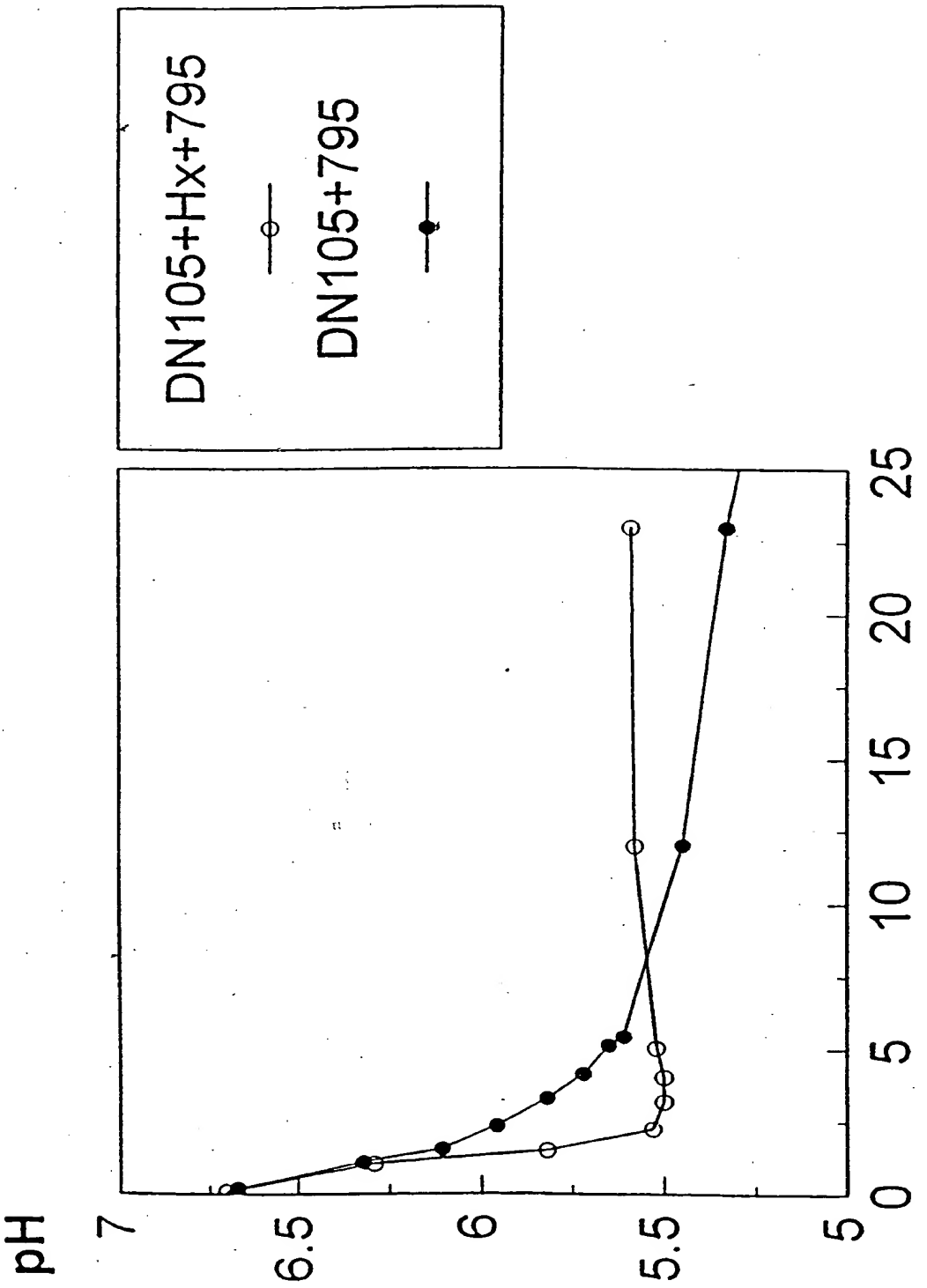

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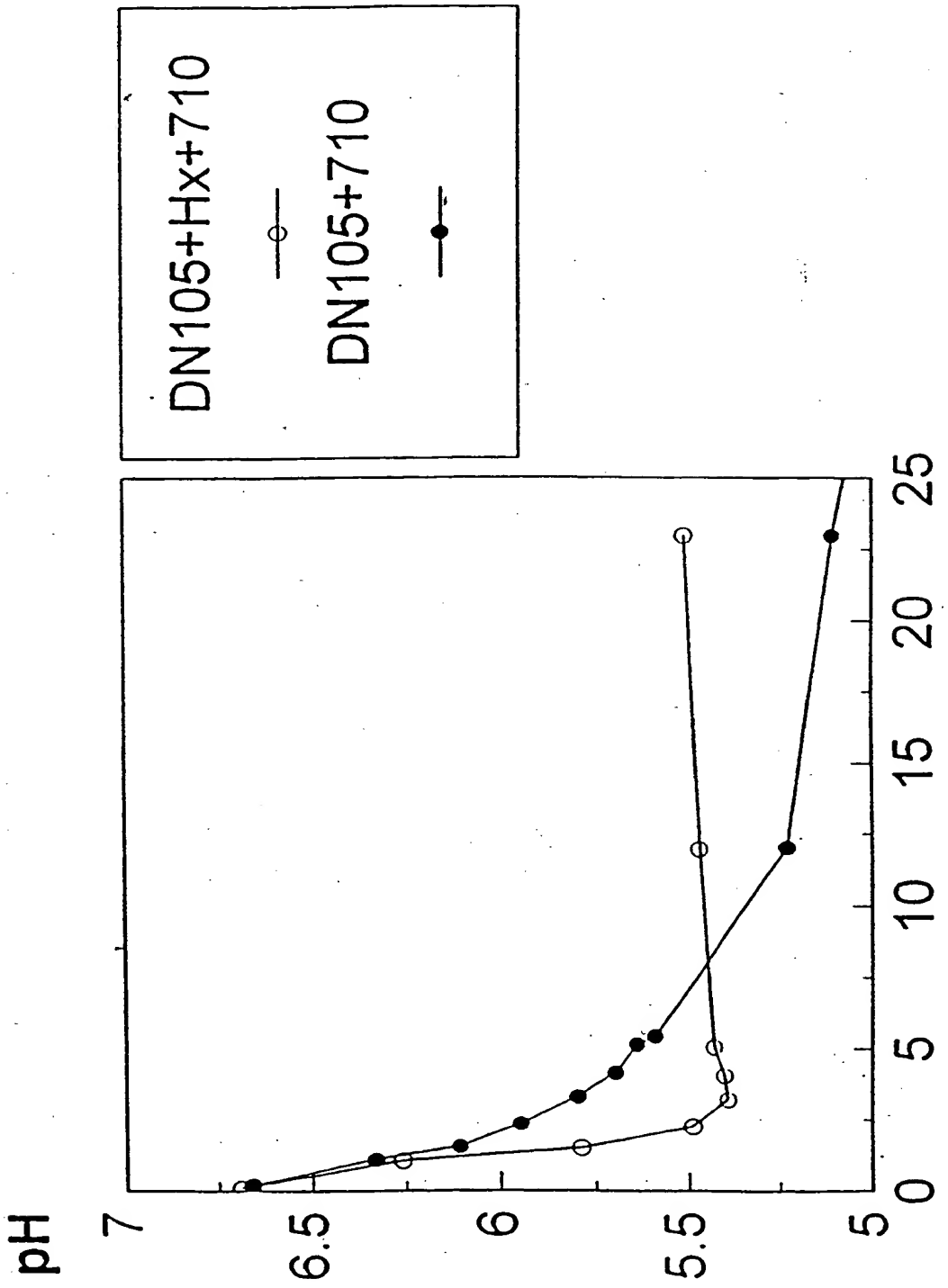

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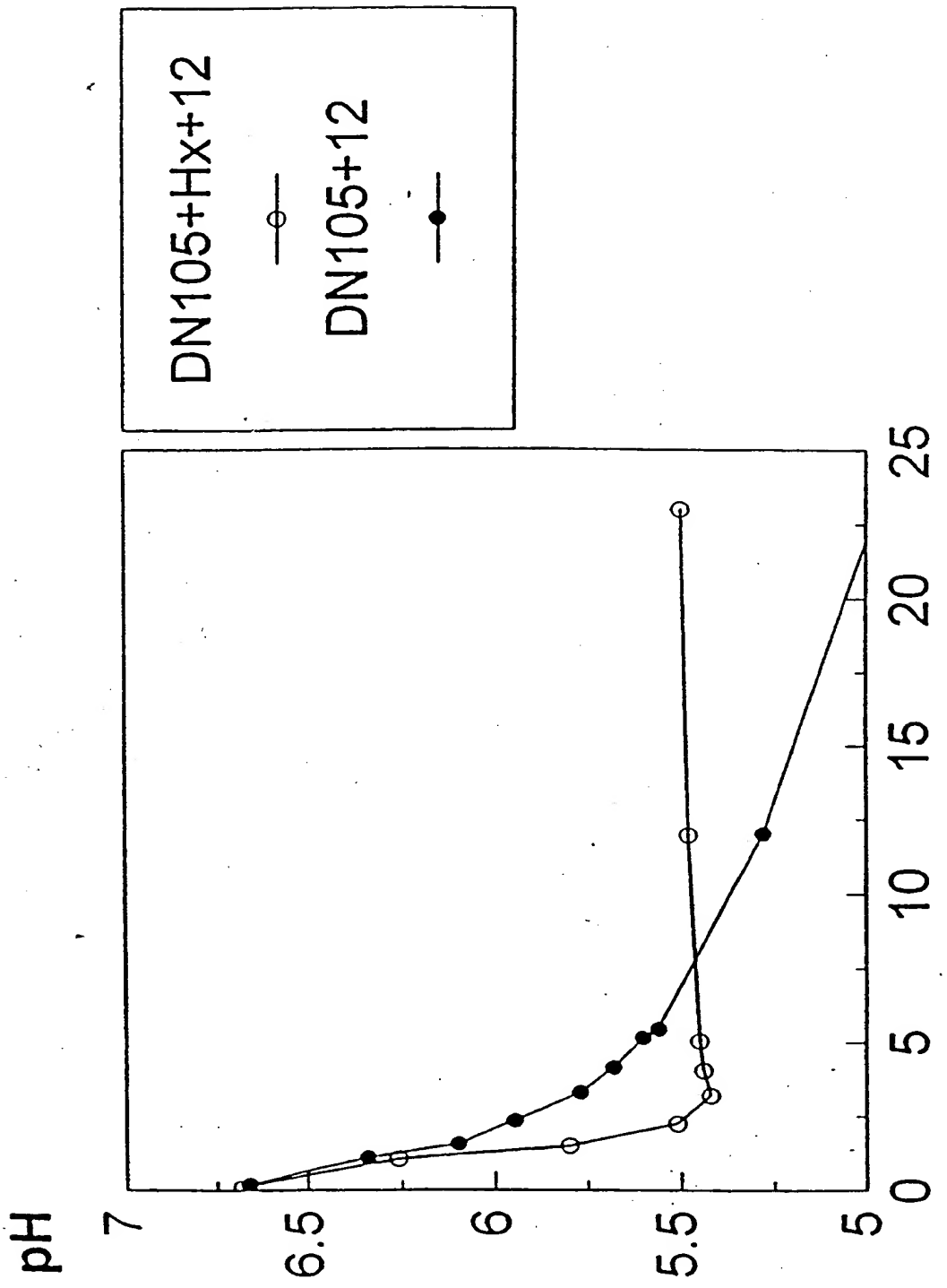

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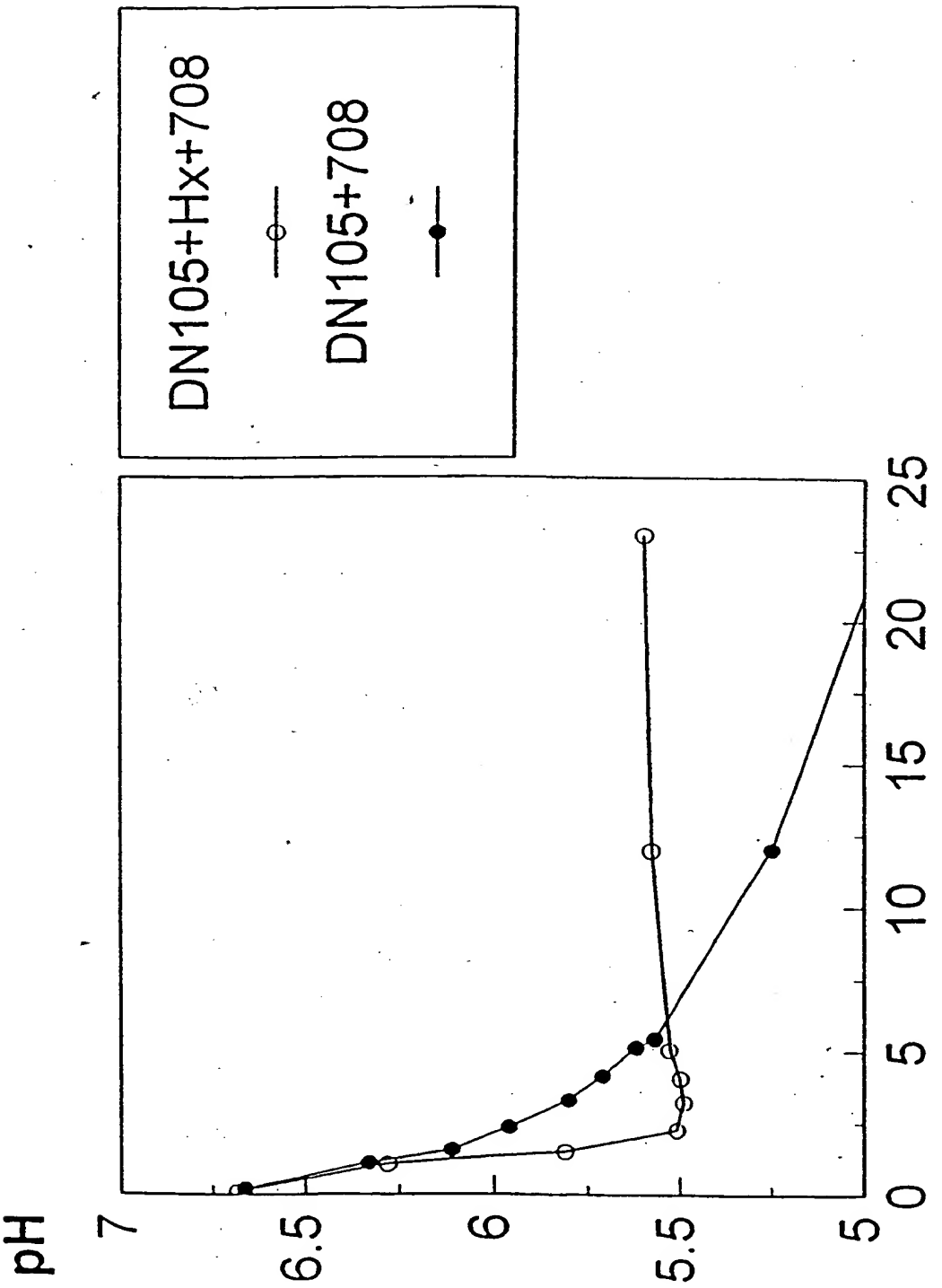

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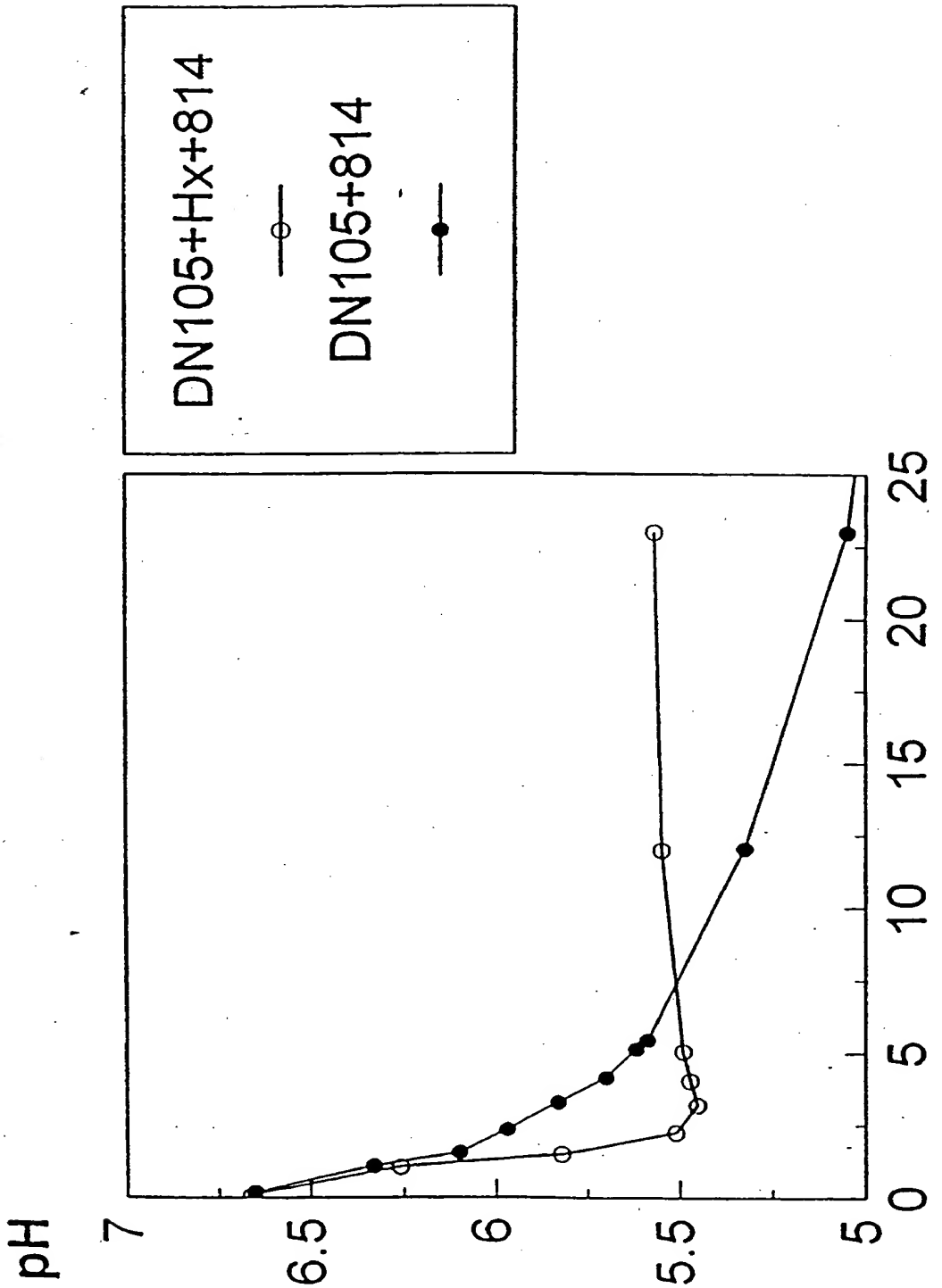

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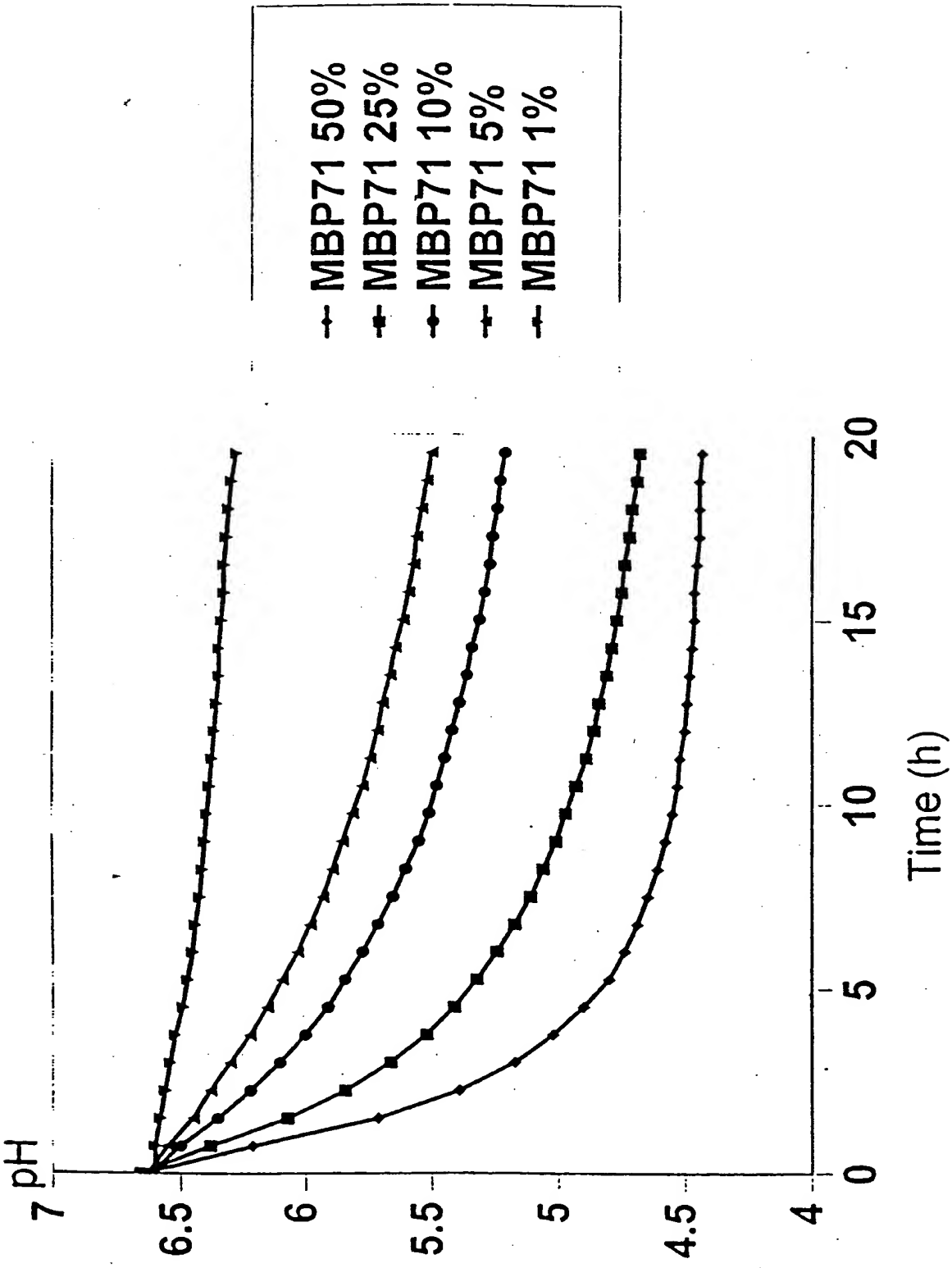
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6
5.5
5
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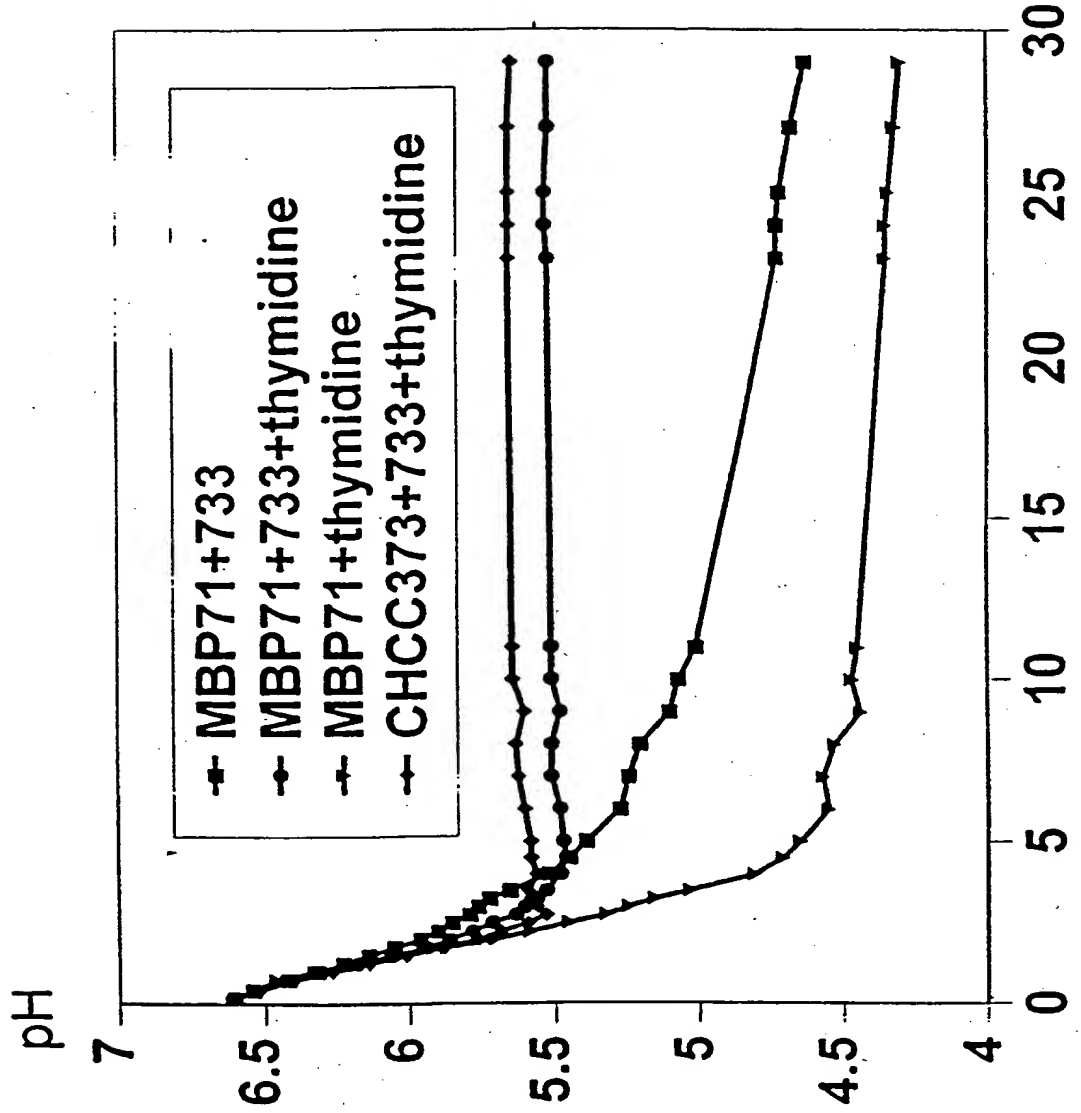
4
0
5
10
15
20
Time (h)

Fig. 4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MBP71+733+thymidine
MBP71+thymidine
CHCC373+733+thymidine
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6
5.5
5
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10
15
20
25
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Time (h)

Fig. 5

SEQUENCE LISTING

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Thomas Janzen

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